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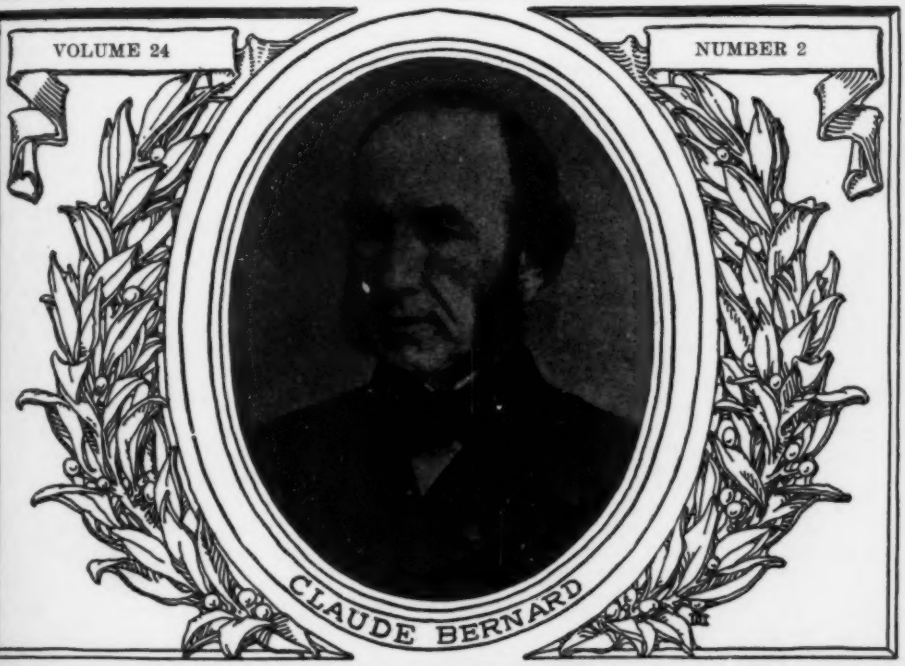

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THE MINIMUM VITAMIN A REQUIREMENT OF THE FOX

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ONE TEXT FIGURE AND ONE PLATE (FIVE FIGURES)

(Received for publication March 12, 1942)

A few experimental studies have been made of the qualitative need of the various vitamins by foxes. This report is the first one setting forth the quantitative need of a vitamin by the fox. It is now known that foxes need vitamin D (Smith and Barnes, '42), anti-grey hair vitamin (Lunde and Kringstad, '39; Morgan and Simms, '40), vitamin B_1 (Green, Carlson and Evans, '41; Carlstrom and Jonsson, '38; Ender and Helgebostad, '39; Hodson and Smith, unpublished data), and nicotinic acid (Hodson and Loosli, '42), and that they do not need vitamin C in their diets (Mathiesen, '39).

Coombes, Ott and Wisnicky ('40) fed ranch fox pups a diet low in vitamin A over a period of 210 days but they observed nothing distinctly abnormal. The diet while low, contained significant amounts of vitamin A. Holmes, Tripp, Ashbrook and Kellogg ('41) studied the liver storage of vitamin A in ranch-raised silver foxes and trapped wild foxes. The former had a range of 1.3 to 10.9 lovibond blue units of vitamin A per gram of liver as compared to an average of 326 blue units in the latter. A preliminary account of vitamin A deficiency in fox pups was made by Smith ('41). The investigations herein reported extend these observations and in addition include the results of studies of the minimum vitamin A requirement of growing pups.

[1] The author is grateful to Dr. L. A. Maynard for helpful advice and interest.

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MATERIALS AND METHODS

This study has extended over a period of 3 years and has involved thirty-four silver fox pups. The first two experiments were concerned with a determination of the qualitative need for vitamin A, which included a detailed study of the deficiency symptoms. In the third year sixteen fox pups were used to determine the minimum vitamin A requirement necessary to prevent the deficiency symptoms in growing pups. The general plan of all of these studies has been to place nursing dams on a diet lacking rich sources of vitamin A so that a large supply of vitamin A would not be built up in the pups. The young were weaned at 6 weeks of age and placed on the experiments.

The basal, vitamin A deficient diet used in these studies had the following percentage composition: meat scrap 30, skim milk powder 7, oatmeal 30, dried yeast [2] 5, cottonseed oil [3] 5, steamed bone meal 2, salt mixture 1 and water 20. To this diet were added irradiated yeast and ascorbic acid, the latter being purely precautionary. The meat scrap (65% protein) and skim milk powder were spread in thin (2 inches) layers and heated at approximately 70° C. for 4 days to insure destruction of traces of vitamin A. The salt mixture consisted of 90% sodium chloride and 10% ferrous sulphate. This diet when fed to 3-week-old rats produced a delayed but characteristic vitamin A deficiency, whereas the control rats supplemented with cod liver oil grew and remained healthy.

The experimental pups were placed in 4-foot by 6-foot wire-floored pens and fed the basal diet ad libitum. Vitamin A supplements when given were administered by mouth as is discussed later. Fresh water was available at all times. Tetrachlorethylene was given as a vermifuge and the pups were treated for ear mites as often as necessary by swabbing out the ears with a mixture of tincture of iodine, ether and glycerol in equal parts. The foxes were weighed at weekly

[2] Product of the Northwestern Yeast Co.
[3] Wesson.

intervals for the first 3 months and thereafter at biweekly intervals. When the foxes died or were killed the livers were tested for vitamin A, using the Carr-Price reaction ('26). Blood for vitamin A determinations was obtained by cardiac puncture. The vitamin A determinations were made by the technic of Kimble ('39), except that a photo-electric colorimeter which had previously been standardized with crystalline vitamin A was used. With the exception of the nervous tissue, the various tissues studied were fixed in Bouin's fluid and stained with hematoxylin and eosin. The nervous tissue was fixed in 10% formaldehyde and stained by a modified Marchi technic (Swank and Davenport, '35). In a few cases the brain was perfused with a potassium bichromate-magnesium sulphate solution before removal. The nervous tissue was imbedded in celloidin and then sectioned.

SYMPTOMS OF VITAMIN A DEFICIENCY

All of the eighteen pups which received the basal vitamin A deficient diet developed a series of nervous derangements which usually began with a trembling of the head. This was followed shortly by what we have termed head "cocking," an action resembling that of a chicken observing a hawk or an airplane flying overhead. The sense of balance was definitely disturbed, for the affected pups, in viewing an object behind them, jerked their heads over their shoulders instead of turning around in the normal fashion. Often in doing this they lost their balance completely and rolled over. In addition, ten of the pups ran rapidly in circles especially when they were excited. This occurred frequently and often lasted for 10 to 15 minutes at a time. The severity of the symptoms varied but appeared to be worst on hot days. The time of onset of the symptoms varied from 1 to 5 months after the start of the experiments, but most of the pups showed the first symptoms within 2 months.

A more delayed symptom (2 to 4 months) which occurred in four of the foxes was that of coma. These pups would appear to be losing their balance, would waver and drop to

the floor and become completely unconscious. Occasionally there was some thrashing of the legs. These seizures lasted usually for 5 to 15 minutes, after which the pups would shakily arise and assume normal attitudes. These comas sometimes occurred spontaneously but usually they followed periods of excitement caused by handling or pounding on the pens.

Six pups were observed for longer periods of time, and of these, three developed typical xerophthalmia (fig. 2) at the eighteenth, twenty-fifth and twenty-seventh week following the start of the experiment. Unilateral at first, this soon affected both eyes in all three cases. These foxes were killed soon after the appearance of xerophthalmia but not before ulceration and rupture of the eyeballs had occurred. Papillary edema was definitely observed in two foxes in the more chronic stages of the deficiency. One female fox came into estrus and was bred. She conceived but aborted a litter of five well-formed fetuses at the forty-third day of pregnancy (normal gestation 52 days).

Microscopic examination of various epithelial tissues did not show any metaplasia as noted in other animals (Wolbach and Howe, '25) until approximately at the time of appearance of xerophthalmia. At this time stratification and keratinization of the epithelium of the cornea, trachea (fig. 3), bronchi, kidney pelvis, urinary bladder and vagina (fig. 4) were observed. Vaginal smears were taken twice weekly from a group of six female pups and in no case did cornified epithelial cells appear until the very late stages of the deficiency. In these animals the nervous symptoms preceded the vaginal cornification by several weeks. The epithelial changes thus appear considerably later than the nervous disorders which are the first clinical symptoms of vitamin A deficiency in the fox. Nerve fiber degeneration was observed in several parts of the nervous system which thus explains the clinical observations. This phase of the study is to be reported in detail elsewhere and only the more salient points will be mentioned here. There was widespread myelin degeneration in the spinal cord (fig. 5) and in the VIII cranial nerve (fig. 6) in which

both the cochlear and vestibular divisions were involved. Mellanby ('38) has made similar observations in vitamin A deficient dogs. The foxes must have been deaf though the clinical detection of this was difficult. This disturbance of the vestibular apparatus undoubtedly explains the upset sense of balance of the pups. Areas of the brain which have so far shown myelin degeneration are the basal ganglia, nuclei of the VIII nerve and many fiber tracts concerned with motor sense. Overgrowth of the cranial and vertebral bones as a contributing factor to the nerve degenerations as observed by Mellanby ('38, '41) in vitamin A deficient dogs was not investigated here.

Liver samples (15 to 35 gm.) of the foxes which received the unsupplemented basal diet in every case failed to show detectable amounts of vitamin A by the Carr-Price reaction, whereas those foxes which received certain levels of vitamin A supplements did show liver storage (table 1).

Attempts to cure the nervous deficiency symptoms have failed, even when 50,000 I. U. of vitamin A were given twice weekly over a period of 3 weeks. Other symptoms of the deficiency, such as loss of appetite and lethargy, were improved by these administrations. Two foxes which developed the nervous symptoms were subsequently placed on a complete diet fed to the stock foxes. Even after a period of 1 year these two foxes still showed nervous disturbances. These results are in keeping with the fact that nerve injuries of the type involved here are irreparable once established.

THE MINIMUM REQUIREMENT

The minimum requirement is defined as the amount of vitamin A, calculated to a daily dose, that just prevents clinical symptoms of vitamin A deficiency. This amount has been found adequate for good growth and general well-being.

On June 2, 1941, sixteen fox pups used in this study were divided into six groups, five of which contained three each. These groups consisted of a negative control group which received the vitamin A deficient diet only; four groups which

received, respectively, 15, 25, 50 and 100 I. U. of vitamin A per kilogram of body weight per day, and a positive control group which, in addition to the basal diet received a cod liver oil concentrate (about 8,000 I. U. of vitamin A per pup per day). With the exception of the last group the vitamin A supplement was U. S. P. reference cod liver oil which was administered by mouth twice weekly. The dose was adjusted after each weekly weighing.

The results are summarized in table 1. The nervous symptoms characteristic of vitamin A deficiency appeared in all members of the negative control group and in two of the three foxes which received 15 I. U. of vitamin A per kilogram per day. All groups receiving more vitamin A than this failed

TABLE 1

The results of the studies of the minimum vitamin A requirement of growing foxes.

GROUP	FOX	START OF DEFICIENCY SYMPTOMS	VITAMIN A IN THE		REMARKS
			Liver	Blood	
		weeks	*µg./100 gm.*	*µg./ml.*	
Negative	46	13	0	..	Killed 12/15/41.
control	40	11	0	..	Moribund 11/26. Killed.
	52	11	0	..	Moribund 10/4. Killed.
15 I. U.[1]	57	25	0	0	Killed 12/15/41.
	53	11	0	0	Killed 12/15/41.
	49	..	0	0	Killed 12/15/41.
25 I. U.	50	..	0	Trace	Killed 12/15/41.
	47	..	Trace	0	Killed 12/15/41.
	54	..	0	..	Died 7/25 from heat collapse.
50 I. U.	55	..	70	Trace	Killed 12/15/41.
	41	..	0	0.34	Killed 12/15/41.
	42	..	..	0.34	Died 12/6. Cause unknown.
100 I. U.	48	..	33	0.55	Killed 12/15/41.
Positive	51	..	125	1.30	Killed 12/15/41.
control	56	..	888	0.77	Killed 12/15/41.
	45	..	825	0.35	Killed 12/15/41.

[1] International units of vitamin A per kilogram of body weight per day.

to show any indication of vitamin A deficiency. The minimum vitamin A requirement thus lies between 15 and 25 I. U. per kilogram of body weight per day.

The vitamin A content of the liver and blood plasma was variable, which is to be expected with such small numbers. The trends, however, are evident. No appreciable liver storage of vitamin A occurred until 50 to 100 I. U. of vitamin A per kilogram per day was administered. The positive control pups which received about 8,000 I. U. per pup per day had large liver stores of vitamin A. Detectable amounts of vitamin A were found in the liver and blood in one fox of the group which received 25 I. U. per kilogram per day, and in increasing amounts in the groups which received higher levels. The three positive control foxes averaged 0.81 μg. of vitamin A per milliliter of blood plasma.

Foxes 40 and 52 were killed in a moribund condition so that the brains could be removed free of postmortem changes. Fox 54 died after being handled on a very hot day in July; the symptoms indicated heat collapse, a condition to which foxes are apparently very sensitive. Fox 42 died in the late stages of the experiment from an unknown cause. This fox gave no clinical indications of vitamin A deficiency and the blood contained significant amounts of vitamin A. Unfortunately the liver sample was lost.

The curve of average growth of the positive control foxes is compared to that of the negative control foxes in figure 1. It will be noted that the growth rate declined in the late stages of vitamin A deficiency, associated with a decreased appetite and listlessness. It was surprising to note, however, that growth was so good in the negative control foxes, the decline not occurring until some weeks after the onset of the nervous symptoms. This was also noted in the experiments of the previous years. The growth of the foxes receiving 25 and 50 I. U. was as good as that of the positive control group.

Of course the fur ranchers are interested in the quality of the furs produced by foxes. The small numbers of animals studied here do not permit a critical comparison of the fur

of vitamin deficient and normal animals. The furs of the six foxes in the negative control group and the 15 I. U. group were not distinctly different, however, from those of the remaining foxes in this study. So far as can be seen from this study vitamin A deficiency has no specific effect on fur quality.

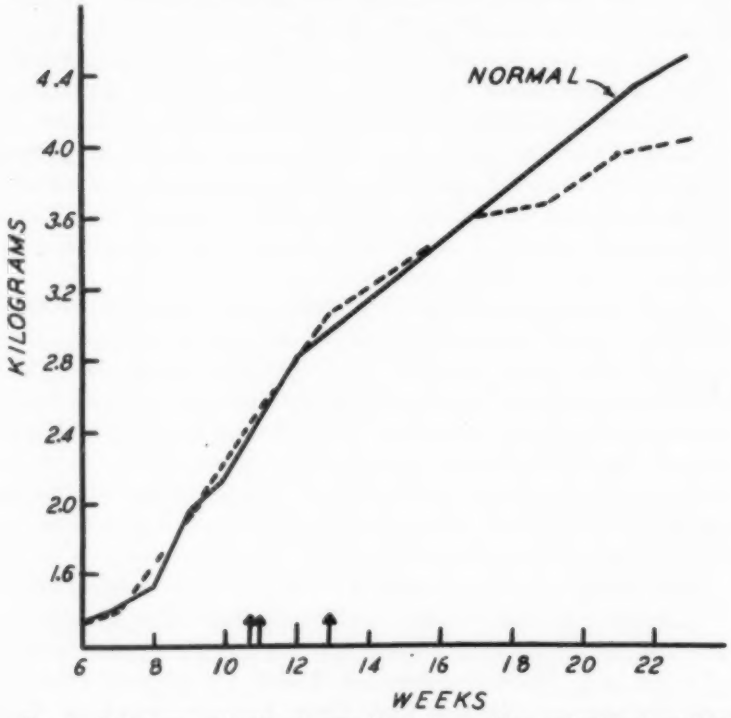

Fig. 1 The curves of average growth of the positive control and negative control foxes. The pups were started on experiment at 6 weeks of age. The arrows indicate the onset of the nervous deficiency symptoms in the negative control foxes.

DISCUSSION

The results of these studies agree well with the minimum vitamin A requirement of cattle, sheep, swine, horses and rats (Guilbert, Howell and Hart, '40). They also lend support to the statement of the foregoing authors that the vitamin A

requirement is more nearly related to the body weight of mammals than to the energy requirements. These authors showed that this observation held for members of the ungulates and rodents whereas this study extends it to a carnivore.

With some slight modifications, the symptoms displayed by vitamin A-deficient foxes have their parallel in symptoms exhibited by other vitamin A deficient animals (Hart and Guilbert, '37; Hart, '40), that is, nervous disturbances, xerophthalmia, abortions, and epithelial metaplasia. Methods for testing night blindness in foxes unfortunately have not been successful. The nervous running in circles was noted also in vitamin A deficient dogs (Frohring, '35).

That a cure of the nervous disturbances did not follow the administration of vitamin A is not surprising for, as Hart and Guilbert stated, "Lesions in nerves once demonstrated are usually permanent and probably often progressive." Zimmerman and Cowgill ('36) were unable to effect a cure of demyelinized nerve fibers caused by vitamin A deficiency in the rat.

Of the farm animals, the vitamin A-deficient pig exhibits a syndrome more nearly like that of vitamin A-deficient foxes. Hughes, Lienhardt and Aubel ('29) stated that, "With swine, we have found the incoordination to be the outstanding symptom of avitaminosis A, while the eye lesions, which are so prominent in the rat, are of little importance." In the fox the neurological symptoms were the earliest and most evident signs of a deficiency of vitamin A.

It should be emphasized that the requirement here defined is a minimum requirement. The requirement for other bodily functions, notably reproduction, is undoubtedly higher. For practical purposes several times the minimum requirement should be fed.

SUMMARY

Experimental vitamin A deficiency in the fox is characterized by nervous disturbances — trembling and "cocking" of the head, periods of whirling and in some cases coma, xeroph-

thalmia, widespread epithelial metaplasia, demyelinization of many nerve fibers and abortions. The earliest signs of a deficiency of vitamin A are the nervous symptoms. The growth of deficient animals while good at first, declined in the late stages. No specific effect of avitaminosis A was noted on the quality of the fur.

The minimum vitamin A requirement necessary to prevent the occurrence of the nervous symptoms in growing pups lies between 15 and 25 I. U. per kilogram of body weight per day. Storage of vitamin A did not occur in the liver until 50 to 100 I. U. of vitamin A per kilogram per day was fed.

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PLATE 1

EXPLANATION OF FIGURES

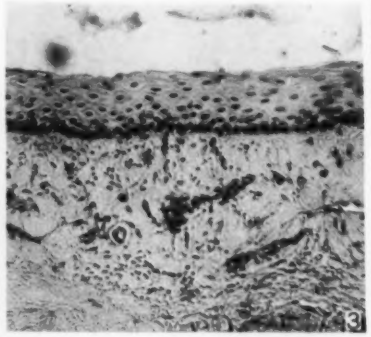
2 Xerophthalmia in a vitamin A deficient fox.

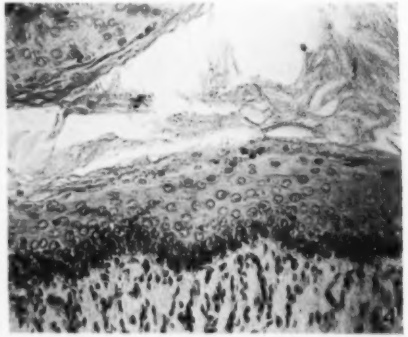
3 This shows a stratified, squamous epithelium lining the bronchus of a vitamin A deficient fox. The normal epithelium is a pseudostratified, ciliated epithelium.

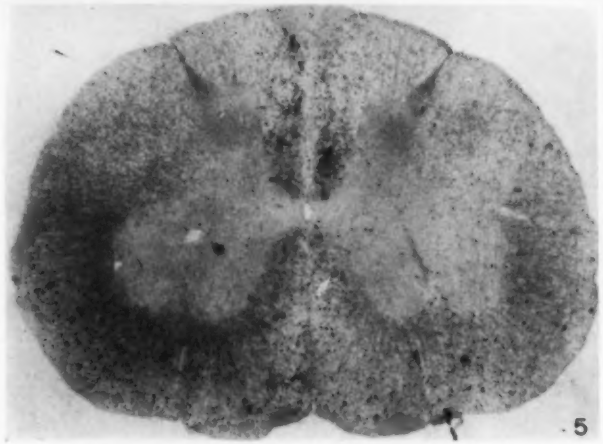
4 This shows the highly stratified, squamous epithelium, much of which is desquamating, in a vitamin A deficient female fox. This section was taken in the fall when vixens are in anestrum. The normal vagina at this time has an epithelial layer consisting of one to three layers of cuboidal cells.

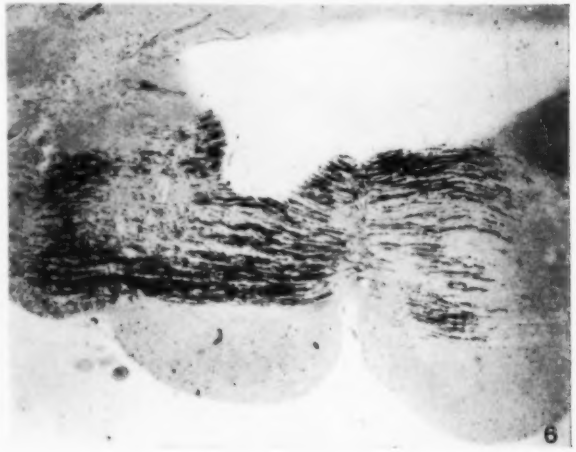
5 Cross-section of the spinal cord of a vitamin A deficient fox showing widespread demyelinization of the nerve fibers. Marchi stain.

6 Cross-section of the medulla oblongata of a vitamin A deficient fox. The section is through the trapezoid body and the demyelinization of the fibers of the VIII nerve is evident. Marchi stain.









THE INFLUENCE OF ALUMINUM SULFATE AND ALUMINUM HYDROXIDE UPON THE ABSORPTION OF DIETARY PHOSPHORUS BY THE RAT [1]

HAROLD R. STREET

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(Received for publication March 18, 1942)

The work of previous investigators has indicated that the feeding of sufficient amounts of soluble aluminum salts under experimental conditions renders much of the phosphorus of the ration non-assimilable. Thus, Cox, Dodds, Wigman and Murphy ('31) have reported that, if aluminum is added to the diets of rabbits or guinea pigs in quantities slightly in excess of that necessary to react with all the phosphorus contained in the ration (as $AlPO_4$), the absorption of phosphorus is practically nil. Deobald and Elvehjem ('35) have described similar results with chickens, using a natural grain ration to which various levels of aluminum salts were added. The addition of aluminum sulfate in amount equivalent to the phosphorus content of the ration resulted in severe symptoms of rickets, with low levels of blood inorganic phosphate. Likewise, Jones ('38) has described the production of marked rickets with low blood inorganic phosphate in rats fed a stock ration with high levels of aluminum sulfate or basic aluminum acetate.

The current use of aluminum hydroxide in the treatment of gastrointestinal disturbances such as peptic ulcer and

[1] This paper was presented in preliminary form at the meeting of the American Physiological Society in Chicago, April 16, 1941 (Street and Barlow).

gastritis has suggested a re-investigation of the effect of aluminum compounds upon phosphorus utilization.

Since this study was completed it has been reported by Fauley and co-workers ('41) that the administration of aluminum hydroxide gel to dogs in amounts equal to twice the phosphorus content of the diet reduced the urinary excretion of inorganic phosphate to approximately half its normal value.

The work reviewed above does not give precise quantitative data as to the effect of aluminum on phosphorus utilization since the degree of availability of the phosphorus in the cereal diets used is unknown (Jones, '39). The experiments described in this paper were designed to determine more quantitatively the influence of aluminum on phosphorus absorption.

METHOD

It was decided to study the utilization of phosphorus by means of the growth rate of young rats placed on a diet adequate except for phosphorus. The addition of sodium phosphate and aluminum compounds in various amounts and ratios to such a diet presumably should permit an accurate evaluation of the effect of aluminum on phosphorus utilization.

The low phosphorus diet of Schneider and Steenbock ('39), slightly modified, was chosen as the most suitable for the purposes desired. By adding phosphate to a diet of this type, which is composed of purified food materials, rations can be constructed in which the phosphorus is present chiefly in known chemical form in contra-distinction to the form and uncertain availability of the phosphorus in the cereal rations formerly used in studies of phosphorus utilization.

The percentage composition of the diet used was as follows: sucrose 44, starch 20, rice bran concentrate [2] 4, dried egg white 18, phosphorus-free salt mixture 4, and cottonseed oil 10. To this was added 0.3 cc. of a 0.3% solution of carotene in oil,

[2] Vitab rice bran concentrate, obtained from the National Oil Products Company, Inc., Harrison, New Jersey.

400 I.U. of vitamin D (viosterol), and 400 μg. of riboflavin per 100 gm. of ration.

The phosphorus content of this ration was reported by Schneider and Steenbock as 0.04%. Their data indicate that young rats placed on the basal ration alone grow very poorly, but the simple addition of sufficient sodium acid phosphate to bring the phosphorus content of the ration to 0.27% completely corrects the deficiency and results in satisfactory growth.

Various dietary formulae were developed from this basal ration by partially replacing the sucrose with sodium acid phosphate, or with both phosphate and aluminum sulfate or hydroxide in definite molecular proportions of aluminum to phosphorus. The phosphorus content of the basal ration was determined by the method of Fiske and Subbarow ('25) after digestion of the food by a wet ashing technique. Various lots of the basal diet were found to contain 0.035 to 0.042% phosphorus; the egg white used as the source of protein in the ration was found to have a phosphorus content of 0.08 to 0.10%. The drinking water used was a spring water of negligible phosphorus content.

The solubility of aluminum hydroxide in dilute acid and, therefore, in the stomach, depends upon its method of preparation. U. S. P. aluminum hydroxide is practically insoluble in tenth-normal hydrochloric acid. This material should be physiologically inert as a component of the diet, since it would not dissolve in the stomach. On the other hand, certain forms of commercial aluminum hydroxide used in clinical medicine are soluble in dilute hydrochloric acid.

The aluminum hydroxide used in this study was a powdered commercial tablet mixture.[3] Its solubility in acid was indicated by the fact that, when placed in tenth-normal hydrochloric acid, it was found to combine with and neutralize 75% of the theoretical amount of acid within a 20-minute period.

[3] Creamalin tablets, obtained from the Alba Pharmaceutical Company, Inc., Rensselaer, New York.

Young rats weighing 40 to 50 gm. were placed on experimental rations in groups of ten. The growth rates were determined at weekly intervals over a 6-week period, after which the animals were sacrificed and the blood inorganic phosphate determined individually by the method of Fiske and Subbarow ('25).

RESULTS

The massed results of feeding three series of rations are shown below in experiments I to III.

Experiment I. In this experiment different groups of animals were fed the basal low-phosphorus ration alone, or the basal ration plus sodium acid phosphate, or phosphate plus aluminum hydroxide, as indicated in table 1.

It will be seen that the animals on the basal ration grew at the rate of about 3 gm. per week, while the rats of groups 2 and 3, with phosphorus levels of 0.24% and 0.60%, respectively, gained approximately 17 gm. per week. With groups 4 and 5 fed a diet containing 0.6% and 3.0% $Al(OH)_3$, respectively, sufficient phosphate was added to the ration to make the value of the atomic ratio of aluminum to phosphorus equal to 1. In other words, the quantity of aluminum present was that calculated as just sufficient to precipitate all the phosphorus as $AlPO_4$, assuming complete interaction. On the basis of the growth curves the percentile formation of insoluble aluminum phosphate which actually occurred was much less than theory, since the animals of both groups 4 and 5 grew at approximately two-thirds the rate of those in groups 2 and 3 receiving optimum phosphorus.

It will be seen that the observed levels of the blood inorganic phosphate of the different groups were approximately proportional to the growth.

The somewhat subnormal growth of the positive controls in this experiment was due to the use of unheated egg white in the ration, thus resulting in early symptoms of egg white damage (Parsons and Kelly, '33). This artifact was proven by two following experiments in which the dietary egg white

was steamed, dried and ground, thus precluding the possibility of egg white damage.

It is clear that the effect of the aluminum feeding on phosphorus absorption in this experiment was much smaller than would be expected from the work of previous investigators. The two following experiments were carried out in an effort to explain this discrepancy.

TABLE 1

Summary of experiments I, II and III.

GROUP NO.	MATERIAL ADDED TO BASAL DIET	PHOSPHORUS CONTENT OF RATION	ATOMIC RATIO Al: P	AVERAGE GAIN IN WEIGHT IN 6 WEEKS	BLOOD INORGANIC PHOSPHORUS, AVER. VALUE
		%		*gm.*	*mg./100 cc.*
Experiment I					
1	None	0.04		19.8	3.7
2	$NaH_2PO_4 \cdot H_2O$	0.24		105.6	8.2
3	$NaH_2PO_4 \cdot H_2O$	0.60		100.6	9.0
4	$NaH_2PO_4 \cdot H_2O$ + 0.6% $Al(OH)_3$	0.24	1:1	70.6	6.0
5	$NaH_2PO_4 \cdot H_2O$ + 3.0% $Al(OH)_3$	1.20	1:1	72.3	
Experiment II					
1	None	0.04		11.1	3.54
2	$NaH_2PO_4 \cdot H_2O$	0.20		128.9	7.15
3	$NaH_2PO_4 \cdot H_2O$	0.60		145.2	8.37
4	$NaH_2PO_4 \cdot H_2O$ + 1% $Al(OH)_3$	0.20	2:1	43.3	3.19
5	$NaH_2PO_4 \cdot H_2O$ + 1% $Al(OH)_3$	0.60	2:3	137.7	7.87
6	$NaH_2PO_4 \cdot H_2O$ + 2.58% $Al_2(SO_4)_3 \cdot 18\ H_2O$	0.24	1:1	11.9	3.66
7	$NaH_2PO_4 \cdot H_2O$ + 2.58% $Al_2(SO_4)_3 \cdot 18\ H_2O$	0.60	2:5	142.4	9.00
Experiment III					
1	None	0.04		4.7	2.40
2	$NaH_2PO_4 \cdot H_2O$	0.10		38.4	2.98
3	$NaH_2PO_4 \cdot H_2O$	0.15		95.9	4.46
4	$NaH_2PO_4 \cdot H_2O$	0.20		140.8	4.88
5	$NaH_2PO_4 \cdot H_2O$ + 1% $Al(OH)_3$	0.20	2:1	46.8	2.98
6	$NaH_2PO_4 \cdot H_2O$ + 1% $Al(OH)_3$	0.60	2:3	161.7	7.08
7	$NaH_2PO_4 \cdot H_2O$ + 0.5% $Al(OH)_3$	0.20	1:1	84.2	3.65
8	$NaH_2PO_4 \cdot H_2O$ + 2.58% $Al_2(SO_4)_3 \cdot 18\ H_2O$	0.24	1:1	17.7	2.27

Experiment II. In this experiment, summarized in table 1, the effect of aluminum sulfate on phosphorus utilization was compared with that of aluminum hydroxide. The growth of groups 2 and 3 indicates that the level of 0.20% phosphorus in the ration is slightly limiting, since a diet containing 0.60% phosphorus permitted a significantly greater growth. The incompleteness of combination of the ingested aluminum hydroxide with phosphate is indicated by the growth of group 4. Since the gain in weight of this group was one-third that of group 2, receiving the slightly limiting level of 0.20 gm. phosphorus per 100 gm. of ration, it may be assumed that the amount of phosphorus remaining available in ration 4 was approximately one-third that of group 2, or 0.067 gm. per 100 gm., indicating that 0.133 gm. of phosphorus per 100 gm. of ration combined with aluminum. Since sufficient aluminum was present to combine with 0.400 gm. phosphorus per 100 gm., the extent of the combination was only one-third of theory. The performance of group 5 indicates that this limiting effect of 1% aluminum hydroxide on growth can be completely eliminated by increasing the phosphorus level to 0.60%.

The data for group 6 show that when a soluble aluminum salt is fed in an amount just equal to the phosphorus content, there is almost complete failure of growth, indicating that most of the phosphorus in the ration has been made unavailable. Here again, the inhibiting effect of aluminum on growth can be prevented merely by increasing the phosphorus content of the ration, since group 7, receiving 0.60% phosphorus in addition to the aluminum sulfate, made optimum growth.

All three groups of rats showing maximal growth, that is, groups 3, 5 and 7, appeared in excellent condition, with smooth, sleek coats, despite the considerable amount of aluminum in the rations of groups 5 and 7. Thus, the only obviously harmful effect of aluminum feeding in these experiments was its interference with phosphorus utilization.

As in experiment I, the blood inorganic phosphate was low in the rats whose growth was seriously limited by the lack of sufficient available phosphorus, while the groups making normal growth exhibited normal levels of blood phosphate.

Experiment III. The results of feeding this series of rations are shown in table 1. This experiment was carried out in order to determine more quantitatively the effect of aluminum hydroxide on phosphorus utilization. From the data of the first four groups, a curve was constructed relating the per cent of available phosphorus in the ration to the growth obtained. Applying this curve to the performance of group 5, receiving 1% aluminum hydroxide, indicates that there was 0.108% available phosphorus in the ration, so that 0.092 gm. of phosphorus per 100 gm. of food, or 46% of the phosphorus present, was rendered unavailable. Since there was sufficient aluminum present to combine with twice the amount of phosphorus actually contained in the ration, it would appear that only 23% of the aluminum was changed to a soluble form so that it could combine with phosphate. Making the same calculation for group 7, the growth attained corresponds to 0.14% of available phosphorus, indicating that 0.06 gm. of phosphorus per 100 gm. of food was rendered unavailable. This is 30% of the phosphorus present in the ration. Since the amount of aluminum present was just sufficient to combine theoretically with all the phosphorus present, this indicates that 30% of the aluminum was converted to a reactive form.

The results obtained with the feeding of aluminum sulfate in this series were similar to those in experiment II. As in preceding experiments, the level of inorganic blood phosphate was approximately proportional to the growth obtained on the various rations.

DISCUSSION

These experiments indicate that when a soluble form of aluminum, as aluminum sulfate, is fed to young rats in amount equal to the phosphorus content of the ration, there is nearly complete precipitation of phosphorus in the intestinal tract.

This conclusion would appear justifiable, since the growth and levels of blood inorganic phosphate obtained under these circumstances are little, if any, greater than those on the basal low phosphorus ration alone. Since aluminum hydroxide is very insoluble in neutral solution, the ability of this form of aluminum to react with phosphate in the intestinal tract is undoubtedly due to its solution by the acid gastric contents, forming aluminum chloride. It follows, then, that the much smaller effect of the hydroxide on phosphorus absorption, as compared to the sulfate, indicates that only a portion of the aluminum hydroxide dissolves in the stomach.

SUMMARY

When aluminum sulfate was fed in amounts chemically equivalent to the phosphorus in the ration, essentially all of the phosphorus was rendered unavailable. On the other hand, when aluminum hydroxide was fed to young rats at levels of 0.5 and 1.0% of the diet about one-third to one-fourth of the aluminum was converted to a form reacting with phosphorus.[4]

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[4] Dick and Eisele ('42) have recently referred to our preliminary report (Street and Barlow, '41) as the basis in part for their statement, "The use of aluminum hydroxide and aluminum sulfate, which recently has become quite widespread, may have deleterious effects. Therapeutic doses may interfere with the absorption of inorganic phosphate from the intestinal tract, thereby deranging the calcium metabolism of the body." This statement is misleading. Aluminum sulfate is not, to our knowledge, used medicinally. The maximal therapeutic dose of aluminum hydroxide gel by normal oral administration recommended by the A.M.A. Council on Pharmacy and Chemistry ('41) would supply not more than 64 cc. in 24 hours. This amount of 5.5% gel would furnish 3.66 gm. of $Al(OH)_3$. If 30% of this were changed to soluble form as indicated by our experimental data it would combine with 0.44 gm. of P, since 1 gm. of aluminum hydroxide is equivalent to 0.4 gm. P. If a dietary contains 1.32 gm. of P, the level recommended by Sherman ('37), the precipitation of 0.44 gm. P leaves 0.88 gm. available, which is the figure given by Sherman as the minimal maintenance level for a 70 kg. adult. Thus, our data do not support the conclusions drawn from them by Dick and Eisele.

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THE ASCORBIC ACID CONTENT OF EWES' BLOOD, COLOSTRUM AND MILK AND THE EFFECT OF ASCORBIC ACID INJECTIONS[1]

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TWO FIGURES

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The ascorbic acid content of ewes' milk is of interest with respect to both human nutrition and the physiology of vitamin C. Ewes' milk, although not extensively used as a food in the United States, is of great importance as an article of human diet both in European countries and on the continents of Asia and Africa. The total annual world production for human consumption, as given by Zorn et al. ('38) is 5032 million liters. Investigations by these same authors of the nutritive value of ewes' milk for infant feeding indicate that normal development results when infants are weaned on ewes' milk or transferred from cows' milk to ewes' milk. Investigations by the Sudbury Laboratory ('40) indicate that ewes' milk can be profitably produced by American farmers.

Rasmussen, Bogart and Maynard ('38) report values ranging from 25 to 40 mg. per quart (2.7–4.2 mg. per 100 ml.) for the ascorbic acid content of ewes' milk, obtained from determinations on samples taken from one ewe at intervals over a 9-week period. Zorn, Richter and Wiener ('38) give 3.18 mg. per 100 ml. as the average value for the ascorbic acid content

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of milk samples taken from five or six East Friesian milk ewes during 16 weeks in 1936 and 23 weeks in 1937. Buruiana ('39) obtained the value 109.6 mg. per liter (10.96 mg. per 100 ml.) using methylene blue titration.

In the present paper results are reported of the determination of the ascorbic acid content of 104 samples of ewes' blood, and of 165 samples of colostrum and milk by titration with 2,6-dichlorophenolindophenol, and the effect of injections of ascorbic acid on the content of the milk and blood.

EXPERIMENTAL PROCEDURE

Twenty bred Hampshire ewes were used. From June through November, 1938, they were on lespedeza and grass pasture. From December 5, 1938, to April 24, 1939, the daily ration per ewe consisted of 4 lbs. of soybean hay or soybean hay and whole soybeans. After lambing, in addition to the hay, a concentrate mixture made up of 2 parts corn, 2 oats, 2 wheat bran, and 1 part cottonseed meal was fed at the rate of 1.5 lbs. per head daily.

Blood samples were taken at 3- to 4-week intervals during the months of October, 1938, through January, 1939. Sixteen of the twenty ewes lambed and from these ewes samples of colostrum and milk were obtained over a period of about 11 weeks during the months of February through May, 1939. During the last 2 weeks of the period feeding was discontinued and the animals were placed on Italian rye grass and crimson clover pasture.

At the end of this time, while still on pasture, ten ewes received intramuscular injections of ascorbic acid in the scapula region. Five of the animals received injections of 2 gm. each and five received injections of 5 gm. each. Samples of milk were taken at the time of injection, 4 hours after injection, and daily for 5 days following the injection. Blood samples were taken 4 hours after and 1 day after injection. Two other ewes were used as controls, milk and blood samples being taken at the same time as were those from the injected animals.

ANALYTICAL PROCEDURE

Blood. The blood was drawn into 1-oz. blue glass bottles containing 20 mg. of sodium oxalate and 10 mg. of sodium cyanide for each 20 ml. of blood. The ascorbic acid determination was made by the method of Satterfield, Perlzweig and Dann ('37). Four milliliters of plasma, free from hemoglobin pigments, were mixed with 6 ml. of reboiled distilled water and 10 ml. of 4% metaphosphoric acid and filtered. Five milliliter portions of the filtrate were placed in 40 ml. capsule vials and titrated with 2,6-dichlorophenolindophenol using a modified Neale-Forbes titration assembly. The dye solution, previously standardized against a solution of pure ascorbic acid, was added until 1 drop produced a faint pink color visible for 30 seconds. Each titration was completed within 3 minutes.

Milk. The colostrum and milk samples were titrated by the method of King ('37). Five milliliters of milk and 2½ ml. of a solution containing 8% acetic acid and 4% metaphosphoric acid were mixed in a 40 ml. capsule vial and titrated until the appearance of a pink color as above.

RESULTS

The ascorbic acid content of blood for individual ewes before lambing, averaged over the period, ranged from 0.43 to 0.82 mg. per 100 ml. of plasma (table 1) with an average value of 0.66 for eighty determinations. Wide variations were observed in the ascorbic acid levels of blood samples from the same ewe taken at the different sampling periods. Values for single samples range from a low of 0.31 to a high of 1.18.

Comparison of these values, 0.43–0.82 mg. per 100 ml., with values reported for other species shows them to be approximately the same as those for the goat, 0.6–0.8, reported by Richmond et al. ('40), and but slightly lower than those for the human on an adequate vitamin C intake, 0.75 or above, reported by Abt, Farmer and Epstein ('36). Wallis ('40) gives the somewhat lower value of 0.320 for cows' blood, and Holmes, Tripp and Satterfield ('39) find the averages for several groups of Rhode Island Red hens to have the some-

what higher values of from 1.061 to 1.906 mg. per 100 ml. of blood plasma.

The ascorbic acid content of colostrum was found to be considerably higher than that of milk. The values for samples of colostrum (table 1) obtained before the lamb was allowed to suckle ranged from 2.01 to 9.94 mg. per 100 ml., the average value being 5.87. The values were found to drop rapidly during the first few days after lambing. After the fifth or sixth day the rapid decrease in ascorbic acid concentration ceased and from this point throughout the remainder of the period of observation, irregular variations were found. The average values for the milk of individual ewes from the sixth day until the end of the period ranged from 0.38 to 1.77 mg. per 100 ml., with an average of 0.80 mg. per 100 ml. (table 1).

This value is considerably lower than the values 2.7–4.2 mg. per 100 ml. (Rasmussen, Bogart and Maynard, '38), 3.18 (Zorn, Richter and Wiener, '38), and 10.96 (Buruiana, '39) reported by others. It should be noted that the animals used were not milk ewes. Also, though there is not full agreement as to the effect of diet on the ascorbic acid content of milk, it should be pointed out that the diet of these animals contained no good source of vitamin C. This value, however, is not greatly different from the values reported for goats' milk by Richmond et al. ('40) of 0.5–2.0. It is somewhat lower than the values of Holmes, Tripp, Woelffer and Satterfield ('40) for cows' milk: 1.776–2.337 for Guernsey and 1.572–2.044 for Holstein.

It is seen that the average ascorbic acid content of colostrum obtained before the lamb suckled was roughly seven times that of milk obtained after the sixth day, and that the ascorbic acid content dropped rapidly during the first 5 or 6 days to a relatively constant value. Figure 1 shows the general trend for the ascorbic acid content of ewes' colostrum and milk. The average curve, fitted by inspection, is given with two individual curves superimposed as an indication of the variation from this normal average curve.

A high value for the ascorbic acid content of cows' colostrum as compared with that of milk was reported by Rasmussen et al. ('36). These authors believed this to indicate a certain storage of ascorbic acid by the cow during the preparturition period. Kon and Watson ('37) found cows' colostrum to contain slightly more ascorbic acid than did milk. Selleg and King ('36) found that the ascorbic acid content

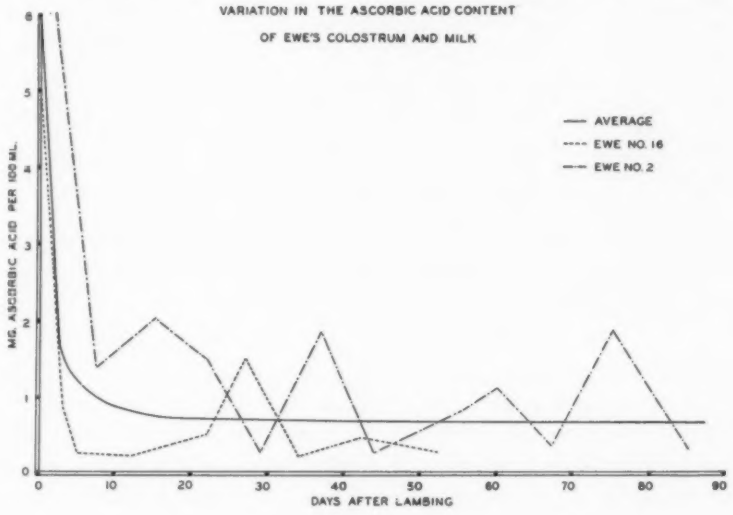

Figure 1

of human colostrum, based upon twenty cases, showed wide variation, but gave a value that was comparable with normal milk. Kasahara and Kawashima ('36) report that the ascorbic acid content of colostrum of Japanese women is 2 to 4 times that of normal breast milk, slowly falling to normal after 3 weeks.

The average values for the ascorbic acid content of blood, colostrum, and milk for each of the animals used is given in table 1.

The twelve animals used in the determination of the effect of injection of ascorbic acid were divided into three groups.

TABLE 1

Ascorbic acid content of ewes' blood, colostrum and milk.

EWE NO.	ASCORBIC ACID IN MG. PER 100 ML. IN			EWE NO.	ASCORBIC ACID IN MG. PER 100 ML. IN		
	Blood [1]	Colostrum [2]	Milk [3]		Blood	Colostrum	Milk
2	0.67	7.73	1.05 (11)	35	0.66	...[5]	...
3	0.65	8.87	1.09 (12)	36	0.59	7.26	0.51 (4)
8	0.66	7.71	0.82 (11)	37	0.52	7.70	0.72 (9)
9	0.76	2.01	0.66 (12)	41	0.64	...[5]	...
13	0.55	4.04	0.93 (9)	42	0.68	...[5]	...
15	0.61	...[4]	0.68 (9)	45	0.71	...[5]	...
16	0.82	5.01	0.49 (7)	46	0.62	6.08	0.68 (2)
18	0.74	5.00	0.54 (10)	49	0.79	4.62	1.77 (19)
28	0.70	3.06	1.06 (10)	51	0.43	3.05	0.54 (7)
29	0.81	5.99	0.94 (11)	982	0.66	9.94	0.38 (9)

[1] Average of four samples taken at 20–25 day intervals, prior to lambing.

[2] Sample collected before lamb was allowed to suckle.

[3] Average of samples obtained after 6th day from lambing. Figures in parentheses indicate number of duplicate determinations included in average.

[4] Colostrum nursed out before sample could be taken.

[5] Did not lamb.

Group I was composed of two animals which received no injections, group II was composed of five animals each of which received an injection of 2 gm. of ascorbic acid, and group III was composed of five animals each of which received an injection of 5 gm. of ascorbic acid. In the group receiving 2-gm. injections the ascorbic acid content of blood samples taken 4 hours after injection was roughly 4 times the normal value, ranging from 2.87 to 3.13; in the group receiving 5-gm. injections, it was roughly 10 times the normal, ranging from 5.95 to 7.93. After 1 day the blood content of animals of both groups had dropped to approximately normal values of from 0.84 to 1.11.

The effect of the injections on the ascorbic acid content of the milk is shown in figure 2. As a result of large variations between animals within groups, a larger number of animals would be required to give more definite conclusions. Individual averages for animals in group II varied from 3.07 to 6.01, and in group III from 3.27 to 6.79 mg. per

100 ml. The increase after injection for individuals varied from 11 to 30% of the initial values in group II and from 15 to 50% of the initial values in group III. The daily group averages which are plotted in figure 2 indicate a fairly rapid rise in the ascorbic acid content of the milk during the first 24 hours after injection and a return to approximately the initial level after 3 days. The average increases of groups II and III were 17.6 and 20.8%, respectively, of the initial average value.

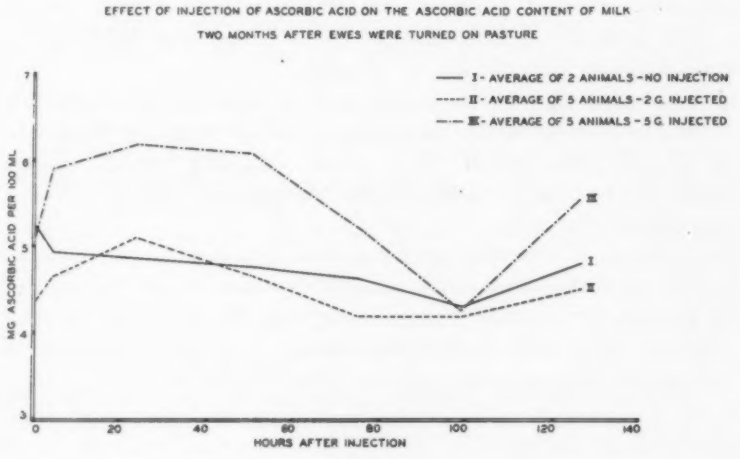

Figure 2

There have been conflicting reports as to the effect of ascorbic acid injections on the concentration of the vitamin in the milk. Sharp ('36) found injections to have no effect on the milk ascorbic acid for the goat; Wendt ('38) noted the same for the cow. On the other hand Richmond et al. ('40) report a small rise in the ascorbic acid content of goats' milk following intraperitoneal injection of 1 or 2 gm. of ascorbic acid. Rasmussen, Bogart and Maynard ('38) report a rise in the ascorbic acid content of milk of one ewe and also of two cows following injections. The present study indicates that injection of ascorbic acid, in this case intramuscularly,

results in a rise in the ascorbic acid content of ewes' milk and a return to normal within 3 days. The magnitude of this rise, however, varies widely with different individuals.

SUMMARY

Results are given of the determination of the ascorbic acid content of 104 samples of ewes' blood and of 165 samples of ewes' colostrum and milk by titration with 2,6-dichlorophenolindophenol, and the effect of injections of ascorbic acid on the content of the milk and blood.

The ascorbic acid content of ewes' blood was found to be 0.43–0.82 mg. per 100 ml. of plasma.

The value for the ascorbic acid content of colostrum obtained before the lamb suckled was found to range from 2.01 to 9.94 mg. per 100 ml. The values dropped rapidly during the first 5 or 6 days to the relatively constant value of 0.80 mg. per 100 ml. for milk.

Injection of ascorbic acid resulted in a rise in the ascorbic acid level in the blood and milk. The level in the blood returned to practically normal within 1 day and the level in the milk within 3 days. There was wide variation in the magnitude of the rise in milk ascorbic acid in different animals.

ACKNOWLEDGMENT

The authors wish to acknowledge their indebtedness to Dr. C. D. Grinnells for assistance in obtaining the blood samples and to Mr. Clarence Early and Mrs. Martha Richmond for technical assistance.

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THIAMINE CLEARANCE AS AN INDEX OF NUTRITIONAL STATUS [1]

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FOUR FIGURES

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Studies of the urinary excretion of thiamine by normal subjects before and after taking test doses, factors influencing such excretion and the changes associated with experimental deficiency have been reported from this laboratory (Melnick, Field and Robinson, '39). On the basis of this work standards were set forth for the interpretation of the urinary thiamine values in the diagnosis of thiamine deficiency. The diagnostic value of such analyses was confirmed by a study of the urinary excretion of thiamine in a large number of clinical cases (Robinson, Melnick and Field, '40).

In the present investigation there is reported a simpler and more reliable procedure for the detection of thiamine deficiency which lends itself readily to routine clinical use.

EXPERIMENTAL PART

Thirty-seven male and female subjects were used in this study. None of these had disorders which might be expected to lead to either faulty absorption, storage, utilization or excretion of the vitamin. Twenty-three were members of the hospital staff and subsisted regularly on what are generally

[1] The expense of this study was defrayed by grants from the Upjohn Company, Kalamazoo, and from the Rackham School of Graduate Studies, University of Michigan.

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regarded as adequate diets. These were considered to be the normal subjects. Fourteen patients having clinical signs or symptoms of thiamine deficiency and giving dietary histories of inadequate thiamine intake constituted the deficient group.

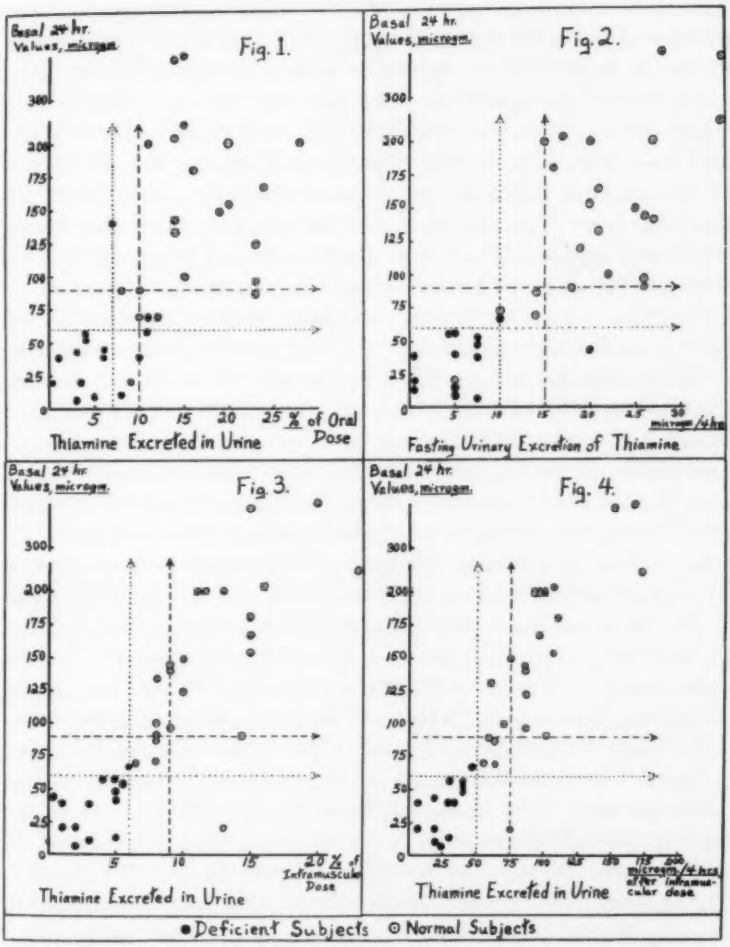
During the 3 days of urinary collections each subject subsisted on a constant diet furnishing at least 900 μg. of thiamine daily. On the first day the basal 24-hour urine sample was collected. On the second day, 12 hours following the last meal, a 4-hour urine sample was obtained from each of the subjects. This is referred to in the present report as the fasting urine sample. Immediately after this period, each subject received parenterally 350 μg. of thiamine per square meter of body area [3] (Peters and Van Slyke, '31). The subsequent 4-hour urine sample was then collected. During these two urinary collection periods, the subjects were in the fasting state. The basal diet was then administered. On the following day and immediately after the largest (midday) meal, a dose of 5000 μg. of thiamine was taken orally.[4] The following 24-hour urine sample was collected. All the urine specimens were then tested for thiamine content according to the colorimetric method of Melnick and Field ('39).

Results of the study are presented in figures 1 to 4. In figure 1 the basal 24-hour urinary thiamine value obtained with each subject is plotted against the per cent of the oral test dose excreted.

In general the correlation is good, confirming our previous reports (Melnick, Field and Robinson, '39; Robinson, Melnick and Field, '40). The deficient subjects excreted small amounts of thiamine, when diet alone was the sole source of the vitamin, and subsequently small fractions of the oral test dose. The

[3] The thiamine solution, containing 500 μg. per cubic centimeter, was administered intramuscularly through a no. 24 needle. Minimal to moderate pressure was applied after the injection.

[4] The necessity for observing these details in collecting 24-hour urine samples, before and after the administration of the oral test dose, was demonstrated by the fundamental studies reported from this laboratory (Melnick, Field and Robinson, '39). Otherwise, variable urinary thiamine values may be obtained which are impossible to interpret.



Figs. 1–4 Correlation between the basal 24-hour urinary thiamine value and the response to the oral test dose of extra thiamine (fig. 1), the 4-hour fasting urinary excretion value (fig. 2), the response to the intramuscular test dose (fig. 3) and the total thiamine excreted during the 4-hour period following intramuscular administration of the vitamin (fig. 4).

normal subjects excreted larger quantities of thiamine in both cases. On each of the graphs (figs. 1 through 4) two additional horizontal and vertical ordinates have been drawn. Those in dotted lines represent what we regard, from both our present and previous studies, as the excretion levels below which thiamine deficiency is invariably found. The ordinates drawn in broken lines represent the bottom limits of the normal urinary thiamine excretion. This level of thiamine excretion is about 50% greater than the basal minimal standards indicated by the dotted lines. The area between the dotted and the broken lines may be regarded as the critical range with respect to thiamine deficiency. This interpretation is supported by the fact that subjects subsisting on diets suboptimal in thiamine intake were shown to fall (Robinson, Melnick and Field, '40) precisely in this range of urinary excretion of thiamine.

It will be noted (fig. 1) that thirteen of the fourteen deficient subjects excreted less than 60 μg. of thiamine in their basal urine samples and that ten of the fourteen excreted less than 7% of the oral test dose. Only one of the twenty-three normal individuals excreted less than the 60 μg. quantity of thiamine in the basal sample. All of the deficient subjects and four of the normal group excreted less than 90 μg. of thiamine in the basal sample. Three of the deficient subjects and twenty of the normal subjects excreted 10% or more of the oral test dose.

In figure 2, the fasting 4-hour urinary excretion of thiamine is plotted against the basal 24-hour values obtained on the previous day. The level of thiamine excretion during the fasting period, associated with deficiency, is considered to be 10 μg. or less; the minimal normal level, 15 μg., 50% greater. The distribution of subjects following this test was found to be comparable to that noted in figure 1. Twelve of the fourteen deficient subjects excreted less than 10 μg. during the fasting 4-hour period; only one of the twenty-three normal subjects excreted in this low range. One deficient subject excreted more than 15 μg. in the fasting sample, while nineteen of the normal subjects excreted in excess of this border-line level.

In figure 3 the per cent of the intramuscular test dose of thiamine excreted in the urine during the first 4-hour period is plotted against the basal urinary thiamine values. The level of excretion, associated with thiamine deficiency, was found to be 6% or less of the test dose. The border line is considered to be 9% of the dose, 50% greater than the minimal level of excretion. Thirteen of the fourteen deficient subjects excreted less than 6% of the intramuscular test dose; none of the normal subjects excreted such a small fraction. All of the deficient subjects excreted less than 9% of the dose. Six of the normal subjects excreted less than this quantity after parenteral administration of the vitamin.

In figure 4 no correction has been made for the fasting urinary excretion of thiamine after the administration of the intramuscular test dose. The total thiamine excretion, in micrograms per 4-hour period, is plotted against the basal 24-hour urinary thiamine value. A distinct difference between the responses of normal and deficient groups is noted. Thus, for the routine diagnosis of thiamine deficiency, it should not be necessary to determine the basal fasting urinary thiamine value. Deficient subjects in the fasting state consistently excrete small amounts of thiamine as well as a small fraction of the test dose. Thus, the over-all effect, as plotted in figure 4, parallels closely the results obtained when the responses, before and after administration of the parenteral test dose, are plotted separately. All of the normal subjects excreted a total quantity of thiamine in excess of 50 μg. during the 4-hour test period after dosage; none of the deficient individuals excreted as much. Six of the twenty-three normal subjects (26%) excreted less than 75 μg. of thiamine. This value, which is 50% above the minimal level of excretion, as in the case of the other graphs, is considered to be critical.

DISCUSSION

No attempt was made in the present study to differentiate between sexes. It has previously been shown that the proportion of parenterally administered (available) thiamine,

excreted in the urine by the normal subject, increases with increasing dosage. This attempt to conserve thiamine when the supply is limited is probably the principal factor responsible for the smaller percentage excretion of ingested vitamin by the normal women subjects (Melnick, Field and Robinson, '39). Additional studies in our laboratory have indicated that when the women subjects ingested diets similar to those eaten by the males, comparable urinary values are obtained. Accordingly, the proposed standards (Melnick, Field and Robinson, '39) for the detection of thiamine deficiency in the case of the female subjects should be revised to coincide with those established for the male. To accept this interpretation, one must conclude that the female subjects whom we considered to be normal were subsisting on borderline levels of thiamine intake. For this group the average daily thiamine intake was approximately 700 μg. At this level of intake the lower urinary excretion indicates an attempt on the part of the body to conserve its supply of thiamine (Melnick, '42).

Confirming an earlier report (Robinson, Melnick and Field, '40), the extent of the urinary excretion of thiamine before and after oral administration of a test dose (5 mg.) was found in the present study to be a reliable index of the nutritional status with respect to this vitamin. Previously (Melnick, Field and Robinson, '39), a preference was indicated for the oral route for administration of the test dose of thiamine. Under such circumstances the rate of excretion is much slower than when the same dose is given by any of the parenteral routes. The organism is rapidly "flooded" with the vitamin after parenteral administration so that the major excretion after dosage in such cases occurs within the first 4-hour period. Such a flooding effect is noted even in patients with obvious thiamine deficiency, and to such an extent that the expected difference in excretion between such individuals and normals is masked. However, in the earlier report comparisons were drawn when 5 mg. doses were administered through the various routes. In the present study, the flooding

effect following intramuscular injection of thiamine was practically eliminated by administration of a small test dose, 0.35 mg. per square meter of body area. (This dose is equivalent to approximately 0.65 mg. for the average male subject.)

There are many advantages in using the response to the parenteral test dose of thiamine both for the diagnosis of thiamine deficiency and for conducting prolonged studies of thiamine metabolism. Errors so commonly encountered in the clinic in the routine collection of 24-hour urine samples are eliminated. It is now necessary simply to collect a 4-hour sample, and this lends itself readily to direct supervision. The parenteral route eliminates variations in rate and degree of intestinal absorption of the vitamin encountered when the oral test dose is taken. The test dose is administered by the clinician and its quantitative intake is not dependent upon cooperation of the patient — a potential source of error.

With this simplified test, it is possible to study "outpatients" during a relatively short visit to the clinic and even to conduct, with a good degree of reliability, field surveys of the extent of thiamine deficiency.

The repeated use of the oral test dose in prolonged studies of thiamine metabolism is in itself a form of therapy. Accordingly, proper interpretation of the results becomes difficult. By occasional use of the small parenteral test dose, averaging a total of 650 μg. of the vitamin, the possibility of significant therapeutic effects being produced is almost eliminated. The omission of the major meal on the day when the parenteral test dose is administered serves to counteract the minimal therapeutic value of the dose so that the nutritional status of the subject during the prolonged study is not significantly changed. Furthermore, the major portion of the small parenteral test dose is excreted within the first 4-hour period so that no carry-over effects are noted.

SUMMARY

Four different thiamine clearance tests were conducted on each of thirty-seven normal and deficient adult subjects.

These consisted of the measurement of the basal 24-hour urinary excretion of thiamine, the fasting 4-hour excretion of the vitamin, the response to the oral administration of 5 mg. of thiamine and the 4-hour excretion of the vitamin when 350 μg. per square meter of body area are administered parenterally. Plotting the results of these tests, one against the other, gave good correlation. The numerous advantages in the use of the parenteral test dose procedure make it the method of choice for studying clinical cases. All normal subjects, but none of the deficient individuals, excreted in excess of 50 μg. of total thiamine during the 4-hour period following dosage.

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VITAMIN B_1 (THIAMINE) REQUIREMENT OF MAN[1]

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THREE FIGURES

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Cowgill ('38) has reviewed the literature up to 1938 dealing with the human requirements for vitamin B_1.

The synthesis of thiamine was soon followed by the publication of chemical and microbiological methods for its determination. Reports from many laboratories using biological assay methods (Harris and Leong, '36; Aoki, '39), the colorimetric procedure (Melnick, Field and Robinson, '39; Hou and Yang, '39; Robinson, Melnick and Field, '40; Cahill, '41; Melnick and Field, '42), the thiochrome method (Westenbrink and Goudsmit, '37; Wang and Yudkin, '40; Najjar and Holt, '40; Carden, Province and Ferrebee, '40; Williams, Mason, Wilder and Smith, '40), and the yeast fermentation procedure (Pollack, Dolger, Ellenberg and Cohen, '40; Youmans, Paton, Kennedy, Monroe and Moore, '40; Pollack, Ellenberg and Dolger, '41 a, b) have shown that the extent of urinary excretion of thiamine is a good index of the nutritional status of the human subject.

The present report is the first attempt through objective thiamine balance studies conducted with humans to determine the vitamin B_1 requirement and the incidence of inadequate

[1] This investigation was begun while the author was Upjohn Fellow in Clinical Research at the University of Michigan. The present paper, summarizing studies conducted at the University Hospital, Ann Arbor, and at the Food Research Laboratories, was reported at the symposium of the American Institute of Nutrition on "Human Requirements for the Vitamins" (Boston, April 1, 1942).

thiamine intake among so-called normal subjects. Some of the material reported herein has appeared in detail elsewhere (Melnick, Field and Robinson, '39; Robinson, Melnick and Field, '40; Melnick and Field, '42) but the implications of the data relative to the thiamine requirement of man were not emphasized.

The colorimetric method of Melnick and Field ('39) was used throughout this investigation for the estimation of urinary thiamine.

More than 175 normal and deficient subjects were used in the urinary excretion studies carried out by the author and his associates. Of this group 116 had no disorders which might be expected to lead to either faulty absorption, storage, utilization or excretion of the vitamin. These persons were used in the thiamine excretion studies summarized in this report. Sixty of the latter individuals, composed of hospital staff members, laboratory technicians and obviously well-nourished hospital patients constituted the normal group. The remaining fifty-six subjects were patients who exhibited clinical signs or symptoms of thiamine deficiency and gave dietary histories of inadequate thiamine intake.

The results obtained in studies (Robinson, Melnick and Field, '40) of the urinary excretion of thiamine by thirty-nine male and thirty-three female subjects subsisting on adequate diets on the days of the test have indicated good correlation between the urinary excretion of thiamine and the adequacy of the dietary regime prior to conducting the tests. The normal individual consuming an adequate diet apparently tends to excrete part of the extra dietary thiamine in the urine. The deficient subject attempts to conserve dietary thiamine to replenish depleted tissue stores, and not waste it by urinary excretion. When a test dose of thiamine was superimposed upon the dietary intake, the extra vitamin was handled in the same manner as that obtained from the diet, in that it was conserved or wasted, depending upon the nutritional status of the subject. Confirmatory evidence obtained with thirty-seven more subjects has been presented in the preceding

paper (Melnick and Field, '42). In all of these studies the normal subjects subsisted on diets furnishing on the average of 1.0 mg. (333 International Units) of thiamine per day.

This tendency of the depleted subject to conserve thiamine is observed in both clinical and experimental thiamine deficiency. The addition of thiamine to the inadequate diet to raise the level of intake to that of the normal group (in the neighborhood of 1 mg.), does not effect a parallel increase in the urinary thiamine excretion for a considerable period

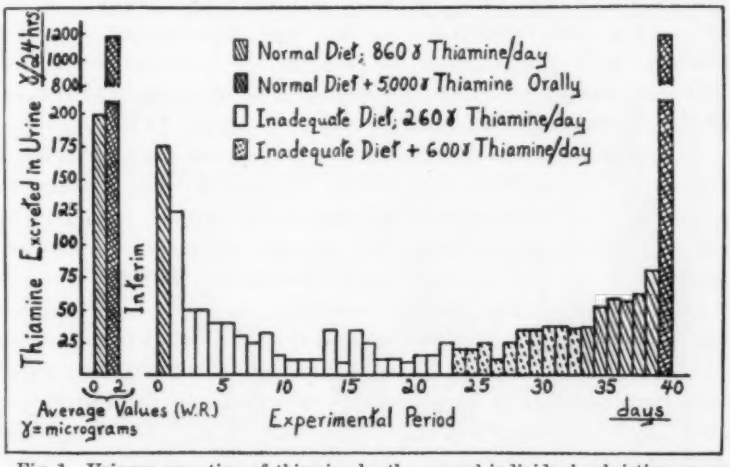

Fig. 1 Urinary excretion of thiamine by the normal individual subsisting on an inadequate thiamine diet and then on the same ration with added vitamin.

of time (Melnick, Field and Robinson, '39; Robinson, Melnick and Field, '40). This is best illustrated by the results plotted in figure 1.

One of the normal subjects, who had been carefully standardized with respect to urinary thiamine excretion while on a constant diet furnishing 860 μg. of thiamine daily, was then fed a low thiamine diet for an extended period. All elements of the diet except thiamine were kept constant. The daily thiamine intake during the depletion period was 260 μg. After the twenty-second day the diet was supplemented with enough

thiamine to equal that of the normal basal ration. The vitamin supplement in aqueous solution was partitioned so that the total thiamine intake at each meal was exactly the same as when the normal diet was ingested.

It will be observed (fig. 1) that the urinary thiamine decreased precipitously from the normal to levels characteristic of avitaminotic individuals. On the twenty-third day, when vitamin supplementation was begun, the body stores of thiamine were significantly reduced. Actually there was apparent definite clinical evidence of deficiency, characterized by aching and tenderness of the calf muscle and Achilles tendon, and paresthesia of the lower extremities. Low thiamine excretion values persisted despite supplementation of the diet with the vitamin.

In order to show that there were no unknown factors associated with the ingestion of the inadequate diet tending to augment the thiamine requirements, the subject returned to his normal basal ration. The urinary thiamine values remained in the subnormal range, indicating that the subject was still conserving dietary thiamine to replenish depleted stores. The percentage of the oral dose excreted in the urine at the end of the study, however, rose to the original normal values. Apparently the thiamine reserves of this individual had been restored by the time the oral test dose was administered.

The difference in thiamine content between the normal and deficient diets in the above study was 600 μg. The addition of that quantity of thiamine to the deficient ration, when clinical symptoms of deficiency were apparent, was without appreciable effect upon the excretion values. However, in the normal subject, such an increment in thiamine intake is promptly followed by an increase in the urinary value of about 100 μg. Analyses of thirty 24-hour urine samples collected over a period of 8 months from two normal male subjects,[2] ingesting adequate diets of varying thiamine content, gave urinary thiamine values paralleling the dietary intakes.

[2] One of these subjects was the same individual used in the study of the urinary excretion of the vitamin during experimental thiamine deficiency.

In one individual the excretion varied from 120 to 220 μg., and in another it ranged from 120 to 260 μg. of thiamine. The range in thiamine intake for the two subjects was from 800 to 1400 μg. per day. Removal of the extra thiamine from the diets of these normal subjects resulted in a return to the basal excretion value within 24 hours. The prompt response of the normal subjects to variations in thiamine intake occurred while they were subsisting on their usual diets furnishing approximately 1 mg. of thiamine per day. Apparently a

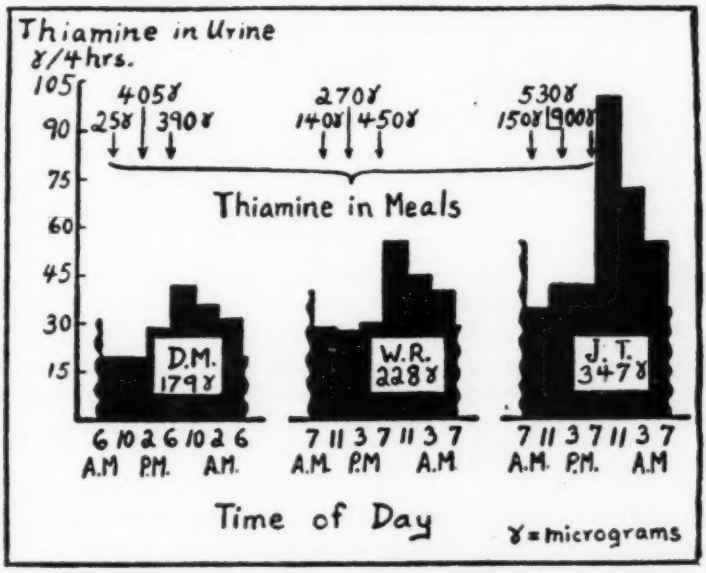

Fig. 2 Normal diurnal variation in urinary thiamine values.

constant daily intake of 1 mg. is sufficient to saturate the subject to such an extent that there is no necessity to conserve extra dietary thiamine.

This conclusion is supported by individual analyses of the six consecutive 4-hour urine samples excreted during a 24-hour period, illustrated graphically in figure 2. A diurnal variation in thiamine excretion is noted in these normal subjects, the extra thiamine being excreted promptly so that by morning

basal minimal figures are obtained. In cases of thiamine deficiency, however, there is no diurnal variation in excretion of the vitamin. The values are minimal and constant.

Subsequent investigations of the urinary excretion of thiamine by normal subjects before, during, and after obvious thiamine saturation have yielded additional evidence in support of the conclusion that a dietary intake of approximately 1 mg. of thiamine per day is adequate for satisfying at least

TABLE 1

Urinary excretion of thiamine by the normal adult before and after thiamine saturation.

SUBJECT	SURFACE AREA	CUSTOMARY DAILY THIAMINE INTAKE	URINARY EXCRETION OF THIAMINE,[1] μG./24 HRS.				
			Basal	Increment after oral test dose [2]	Increment during saturation [3]	"Basal"	Increment after oral test dose [2]
	sq.m.	*μg.*					
D. C.	1.60	870	130	950	4170	339	825
F. O.	1.64	950	150	750	4170	269	650
J. C.	1.75	840	138	1025	5230	337	1000
D. M.	1.88	1060	208	925	4080	370	775
M. H.	1.96	1370	125	1040	4420	308	775
L. S.	2.18	1630	118	700	4000	276	650
Average	1.84	1120	145	898	4350	317	779

[1] On the day that urine samples were collected, each subject received a well-balanced diet furnishing 1070 μg. of thiamine per 2700 calories.

[2] Five milligrams of thiamine taken orally after dinner.

[3] Ten milligrams of thiamine were taken orally each day, 3.33 mg. after each meal, for a period of from 3 to 6 weeks.

the minimal requirement of the adult. The more important features of the study are presented in table 1.

On each of the days of urinary collections the individuals were fed the same constant adequate diet furnishing in each case 1070 μg. of thiamine.[3] The 24-hour urinary thiamine

[3] The thiamine values for the other diets used in the present study were based upon the tables compiled by Williams and Spies ('38). In this particular case, the foods were frozen in solid carbon dioxide immediately after cooking, ground to a uniform mixture and aliquots taken for thiamine analyses according to a modification of the thiochrome method of Hennessy ('41). The figure obtained agreed with that calculated from the tables of Williams and Spies ('38). This gives added support to the reliability of the thiamine values estimated for the remaining diets.

values for the six subjects before and after the ingestion of an oral dose of 5 mg. of the vitamin were all well within the normal range (Melnick, Field and Robinson, '39; Robinson, Melnick and Field, '40; Melnick and Field, '42). During a 3 to 6 weeks' period of thiamine saturation 10 mg. of extra thiamine were ingested daily, 3.33 mg. after each meal. The 24-hour urine samples were collected at the end of each week. Within 1 week the subjects were excreting more than 4 mg. of thiamine in excess of their basal output. This indicates that the organism can excrete large fractions (40 to 50%) of dietary thiamine if a sufficient excess is available. During the remaining period of vitamin saturation, the urinary values remained relatively constant at this high level of excretion. When dosage with extra thiamine was stopped, a precipitous drop in the 24-hour urinary excretion values occurred within the first 4 days to approximately 400 μg. followed by further gradual decrease. Previous tests (Melnick, Field and Robinson, '39) have shown that fully 2 weeks are required before the 24-hour urinary values return to the previous basal level. In the present study, however, the subjects were given the oral dose of 5 mg. of thiamine 7 days after termination of the saturation period. The high "basal" excretions during the 24 hours prior to the administration of the oral test dose constitute good evidence that the six subjects were still in a state of thiamine saturation. The use of this "basal" value in determining the increment of thiamine excretion after the oral test dose was justified since at this period following saturation the return to the normal level is very gradual. The total urinary thiamine figures following administration of the test dose to the saturated subjects were slightly higher than those obtained prior to saturation. The greater values, however, were due entirely to the higher "basal" values noted during this test period. After corrections were made for these elevated "basal" figures, the values were found to be no greater than those obtained in the initial thiamine clearance tests. Because of the possibility that the urinary excretion of extra thiamine following its oral administration to a subject in the saturated state might be slower than that noted in the

first thiamine clearance test, analyses were made of the urine collected during the second 24-hour period following dosage. These showed the same small carry-over values as were observed in the initial tests; viz., 1 to 2% of the oral test dose. Apparently, the thiamine stores of these normal subjects subsisting regularly on diets furnishing approximately 1 mg. of thiamine daily were in no significant state of depletion prior to the saturation regime. Otherwise, a greater degree of thiamine clearance, following administration of the oral dose, should have occurred during the latter test when the subjects were known to be in a state of thiamine saturation. That this deduction is valid is indicated by similar tests conducted on subjects who subsisted previously on diets low in thiamine. The thiamine clearance values were much greater (sixfold) when the tests were conducted after the period of thiamine saturation (Robinson, Melnick and Field, '40).

In a previous study [4] (Melnick, Field and Robinson, '39) on the excretion of thiamine by normal subjects, the average 24-hour urinary value for the males was found to be approximately 200 μg.; the dietary intake was about 1000 μg. In the case of the normal women used in this study, the average daily urinary excretion was approximately 90 μg. The dietary intake, however, was not proportionately less, but in the neighborhood of 700 μg. This is considered to be good evidence that when the dietary intake is 700 μg., there is an attempt on the part of the organism to conserve its thiamine intake and not waste it by urinary excretion. The 700 μg. intake of thiamine may then be regarded as border-line with respect to the normal daily requirement. Support for this is indicated by the fact that when both the normal men and women are furnished extra thiamine (as the oral test dose) they excreted approximately the same fraction, about 13%, of this additional thiamine. In other words, the women were not sufficiently deficient, despite their low thiamine intake,

[4] In this particular investigation the normal subjects, male and female, subsisted on their customary, supposedly adequate, diets of their own choosing.

to show evidence of conservation of the thiamine when an excess became available.

It is of interest to point out that these normal subjects on diets furnishing from 2000 to 3000 calories daily required from 700 to 1000 μg. of thiamine before the above objective signs of conservation of dietary thiamine were apparent. Calculations based upon Cowgill's formula ('34), derived in studies conducted before synthetic thiamine became available, indicate that a dietary vitamin to calorie ratio of 2.0 (or a thiamine to calorie ratio [5] of 0.3) should protect against symptoms of beriberi. Such a vitamin to calorie ratio is synonymous with a dietary intake of 600 to 900 μg. for individuals subsisting on 2000 to 3000 calories per day. This is in excellent agreement with the critical 700 to 1000 μg. range, derived from the urinary excretion studies. The values based upon Cowgill's formula should be expected to be slightly less, since his criterion was protection against the appearance of frank deficiency symptoms.

The value derived from the urinary excretion studies of approximately 350 μg. of thiamine per 1000 calories may be regarded as the minimal daily requirement. If, as is generally done in drawing up other nutritional standards, a 50% margin of safety is allowed, the recommended daily intake becomes approximately 500 μg. per 1000 calories. The validity of these minimal and recommended values for thiamine intake is also supported by the studies of Keys and Henschel ('42), which indicated no improvement, following thiamine supplementation, in the physiological and biochemical responses of active adult males subsisting regularly on diets furnishing 430 μg. of the vitamin per 1000 calories.

As a corollary to the present report, it is of interest to estimate how many of the so-called normal subjects actually subsist upon inadequate thiamine intakes. Figure 3 presents a summation of the results of the studies conducted with the

[5] One Cowgill vitamin unit, the milligram equivalent, is equal to 0.15 μg. of thiamine.

116 subjects at the University Hospital, Ann Arbor, and at the Food Research Laboratories.

These subjects are first classified as deficient, border-line, or normal on the basis of clinical signs, symptoms, and dietary histories. The responses of these subjects to the four types of urinary excretion tests are also indicated. Examination

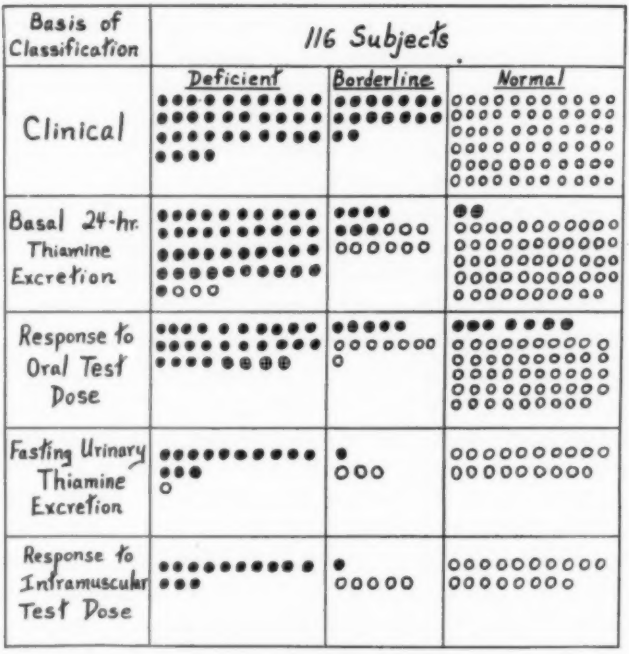

Fig. 3 Correlation between thiamine clearance tests and diagnoses of nutritional status, based upon clinical examination.

of the findings demonstrates that there is a tendency for the clinician to be conservative in his classifications. This is undoubtedly due to the difficulty in detecting sub-clinical or latent signs of thiamine deficiency even by those specially trained in the diagnosis of the avitaminoses. Subjects clinically regarded as deficient, were invariably found to be so according

to results obtained by the four types of laboratory tests; those classified as border-line showed a tendency to excrete minimal quantities of thiamine characteristic of the deficient group, while normal subjects frequently excreted border-line, or even lower quantities of thiamine.

The data used in figure 6 were reassembled in table 2, since not all the subjects were used in all the tests. Some, classified as normal, gave border-line urinary values in more than one type of test; others in only one. For purposes of determining adequacy of nutrition, the normal individual should be regarded as one who passes all of the laboratory tests.

TABLE 2

Nutritional status of subjects used in the present study.

NUMBER OF SUBJECTS	CLINICAL CLASSIFICATION	INTERPRETATION OF CLEARANCE TESTS			
		Malnourished		Normal	
		Number	Per cent	Number	Per cent
34	Deficient	34	100	0	0
16	Border-line	14	87	2	13
66	Normal	18	27	48	73

The results presented in table 2 indicate that the urinary thiamine values of all subjects classified as deficient by clinical criteria were well below the critical range of excretion. Only two of the sixteen border-line cases fell within the normal range in both types of laboratory tests to which they were subjected. Fully eighteen of the sixty-six normal subjects gave low thiamine clearance values in one or more of these tests, as though they were border-line or deficient subjects. Thus, only 73% of the normal subjects were excreting well enough in the normal range to pass all four criteria. This is a surprisingly low percentage inasmuch as these normal subjects consisted mainly of staff members of the hospital or of laboratory personnel whose diets were in no way restricted. However, this high incidence of failures among our so-called normal subjects to pass all the clearance tests may be ex-

first thiamine clearance test, analyses were made of the urine collected during the second 24-hour period following dosage. These showed the same small carry-over values as were observed in the initial tests; viz., 1 to 2% of the oral test dose. Apparently, the thiamine stores of these normal subjects subsisting regularly on diets furnishing approximately 1 mg. of thiamine daily were in no significant state of depletion prior to the saturation regime. Otherwise, a greater degree of thiamine clearance, following administration of the oral dose, should have occurred during the latter test when the subjects were known to be in a state of thiamine saturation. That this deduction is valid is indicated by similar tests conducted on subjects who subsisted previously on diets low in thiamine. The thiamine clearance values were much greater (sixfold) when the tests were conducted after the period of thiamine saturation (Robinson, Melnick and Field, '40).

In a previous study [4] (Melnick, Field and Robinson, '39) on the excretion of thiamine by normal subjects, the average 24-hour urinary value for the males was found to be approximately 200 μg.; the dietary intake was about 1000 μg. In the case of the normal women used in this study, the average daily urinary excretion was approximately 90 μg. The dietary intake, however, was not proportionately less, but in the neighborhood of 700 μg. This is considered to be good evidence that when the dietary intake is 700 μg., there is an attempt on the part of the organism to conserve its thiamine intake and not waste it by urinary excretion. The 700 μg. intake of thiamine may then be regarded as border-line with respect to the normal daily requirement. Support for this is indicated by the fact that when both the normal men and women are furnished extra thiamine (as the oral test dose) they excreted approximately the same fraction, about 13%, of this additional thiamine. In other words, the women were not sufficiently deficient, despite their low thiamine intake,

[4] In this particular investigation the normal subjects, male and female, subsisted on their customary, supposedly adequate, diets of their own choosing.

to show evidence of conservation of the thiamine when an excess became available.

It is of interest to point out that these normal subjects on diets furnishing from 2000 to 3000 calories daily required from 700 to 1000 μg. of thiamine before the above objective signs of conservation of dietary thiamine were apparent. Calculations based upon Cowgill's formula ('34), derived in studies conducted before synthetic thiamine became available, indicate that a dietary vitamin to calorie ratio of 2.0 (or a thiamine to calorie ratio [5] of 0.3) should protect against symptoms of beriberi. Such a vitamin to calorie ratio is synonymous with a dietary intake of 600 to 900 μg. for individuals subsisting on 2000 to 3000 calories per day. This is in excellent agreement with the critical 700 to 1000 μg. range, derived from the urinary excretion studies. The values based upon Cowgill's formula should be expected to be slightly less, since his criterion was protection against the appearance of frank deficiency symptoms.

The value derived from the urinary excretion studies of approximately 350 μg. of thiamine per 1000 calories may be regarded as the minimal daily requirement. If, as is generally done in drawing up other nutritional standards, a 50% margin of safety is allowed, the recommended daily intake becomes approximately 500 μg. per 1000 calories. The validity of these minimal and recommended values for thiamine intake is also supported by the studies of Keys and Henschel ('42), which indicated no improvement, following thiamine supplementation, in the physiological and biochemical responses of active adult males subsisting regularly on diets furnishing 430 μg. of the vitamin per 1000 calories.

As a corollary to the present report, it is of interest to estimate how many of the so-called normal subjects actually subsist upon inadequate thiamine intakes. Figure 3 presents a summation of the results of the studies conducted with the

[5] One Cowgill vitamin unit, the milligram equivalent, is equal to 0.15 μg. of thiamine.

116 subjects at the University Hospital, Ann Arbor, and at the Food Research Laboratories.

These subjects are first classified as deficient, border-line, or normal on the basis of clinical signs, symptoms, and dietary histories. The responses of these subjects to the four types of urinary excretion tests are also indicated. Examination

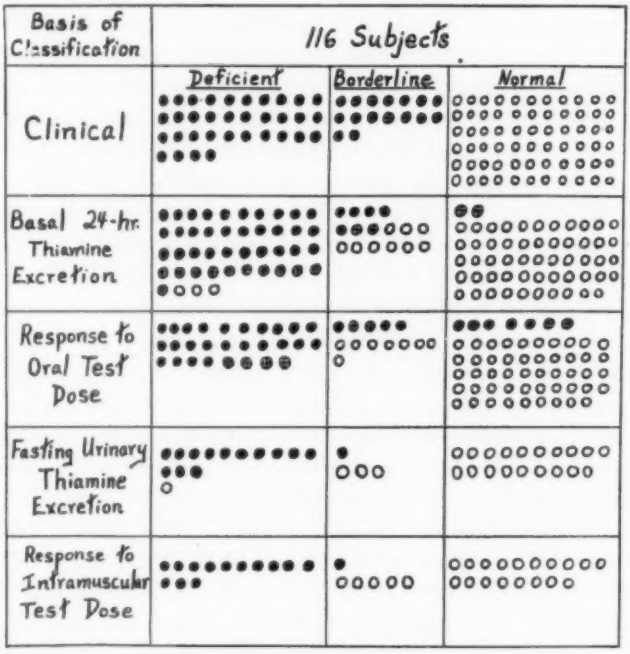

Fig. 3 Correlation between thiamine clearance tests and diagnoses of nutritional status, based upon clinical examination.

of the findings demonstrates that there is a tendency for the clinician to be conservative in his classifications. This is undoubtedly due to the difficulty in detecting sub-clinical or latent signs of thiamine deficiency even by those specially trained in the diagnosis of the avitaminoses. Subjects clinically regarded as deficient, were invariably found to be so according

to results obtained by the four types of laboratory tests; those classified as border-line showed a tendency to excrete minimal quantities of thiamine characteristic of the deficient group, while normal subjects frequently excreted border-line, or even lower quantities of thiamine.

The data used in figure 6 were reassembled in table 2, since not all the subjects were used in all the tests. Some, classified as normal, gave border-line urinary values in more than one type of test; others in only one. For purposes of determining adequacy of nutrition, the normal individual should be regarded as one who passes all of the laboratory tests.

TABLE 2

Nutritional status of subjects used in the present study.

NUMBER OF SUBJECTS	CLINICAL CLASSIFICATION	INTERPRETATION OF CLEARANCE TESTS			
		Malnourished		Normal	
		Number	Per cent	Number	Per cent
34	Deficient	34	100	0	0
16	Border-line	14	87	2	13
66	Normal	18	27	48	73

The results presented in table 2 indicate that the urinary thiamine values of all subjects classified as deficient by clinical criteria were well below the critical range of excretion. Only two of the sixteen border-line cases fell within the normal range in both types of laboratory tests to which they were subjected. Fully eighteen of the sixty-six normal subjects gave low thiamine clearance values in one or more of these tests, as though they were border-line or deficient subjects. Thus, only 73% of the normal subjects were excreting well enough in the normal range to pass all four criteria. This is a surprisingly low percentage inasmuch as these normal subjects consisted mainly of staff members of the hospital or of laboratory personnel whose diets were in no way restricted. However, this high incidence of failures among our so-called normal subjects to pass all the clearance tests may be ex-

plained by the recent observation of Lane, Johnson and Williams ('42) that the average American diet, prior to the advent of enriched bread and flour, furnished only 320 μg. of thiamine per 1000 calories.

SUMMARY

On the basis of objective thiamine balance studies, the vitamin B_1 requirement of the adult is estimated to be 350 μg. per 1000 calories. The recommended daily intake is regarded as 500 μg. per 1000 calories. Only 73% of the so-called normal subjects, who were not restricted as to choice of diet, excreted sufficient quantities of thiamine in the urine to pass all the thiamine clearance tests.

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THE NICOTINIC ACID CONTENT OF MEAT

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The literature now contains a considerable number of observations on the nicotinic acid content of meat; references to these have been collected in the table of Bacharach ('41) and the monograph of Waisman and Elvehjem ('41). Nevertheless, further work is still required, since some of the reported observations are based on analytical methods which are open to criticism (Dann and Handler, '41) and also because most of the figures reported are based on the analysis of a single sample of the particular meat.

In this paper we present the results of analyses of certain commonly-used meats, using samples purchased in local retail stores, together with data on the effect of cooking upon the nicotinic acid content of some of them. The analyses were made by a chemical method previously described (Dann and Handler, '41), in which digests of the samples are treated with Lloyd's reagent and lead hydroxide to decolorize them completely, and nicotinic acid in the colorless extract is then determined by the König reaction using cyanogen bromide and metol. The results are given in micrograms of nicotinic acid per gram fresh weight of meat. In examining the effect of cooking, dry weights of the raw and cooked meats were determined and the nicotinic acid content is expressed on the dry weight basis. This method is subject to error when fat is rendered from the meat during cooking, but the error will be in the direction of increasing the apparent nicotinic acid content of the cooked meat and so decreasing the apparent

loss due to cooking. Therefore, the losses of nicotinic acid from lean meats which lose fat during cooking, such as pork chops, will actually be greater than the figures reported here. The sampling for experiments on the effects of cooking was performed by cutting out a small piece (5 to 10 gm.) of tissue with scissors before cooking and removing a second sample from the tissue close by the first excision after the meat was cooked.

RESULTS AND DISCUSSION

The data obtained are given in the tables. They may be compared with the considerable series of meats assayed at the University of Wisconsin by three different methods, namely,

TABLE 1

Nicotinic acid content of meat as purchased. The mean value is accompanied by its standard error when the number of samples is greater than 5.

MEAT	NUMBER OF SAMPLES	NICOTINIC ACID PER GRAM FRESH WT.	
		Mean	Range
		μg.	*μg.*
Beef liver	8	100 ± 7.4	76–143
Beef kidney	5	77	65–86
Beef muscle meats	17	57 ± 3.4	40–82
Lamb kidney	2		93 and 96
Lamb heart	6	63 ± 2.2	58–72
Lamb muscle meats	11	77 ± 3.9	54–102
Pork liver	11	140 ± 6.5	97–166
Pork muscle meats	14	61 ± 5.3	41–102
Ham	11	50 ± 5.7	24–90
Veal liver	3	123	115–136
Veal kidney	4	90	83–100
Veal muscle meats	8	73 ± 4.3	49–91
Chicken liver	15	152 ± 5.2	114–178
leg meat	15	72 ± 1.8	61–80
breast meat	15	151 ± 5.7	110–181
Rabbit liver	2		221 and 215
kidney	2		130 and 162
leg meat	2		126 and 129
Trout	1		35
Halibut	1		30
Haddock	1		9
Perch	1		17
Scallop	1		14

bio-assay with the dog (Waisman et al., '40), chemical determination by a variant of the König reaction (McIntire et al., '41), and microbiological assay with Lactobacillus arabinosus 17–5 (Waisman and Elvehjem, '41). The values obtained with the Wisconsin samples by microbiological assay all fall within or just outsides the ranges we have observed. In general the same samples showed a higher apparent nicotinic acid content when analyzed by the chemical method employed by McIntire et al. ('41) and a still higher apparent nicotinic acid content when the dog bio-assay was performed.

TABLE 2

Effect of cooking on nicotinic acid content of meats.

MEAT AND MODE OF COOKING	NICOTINIC ACID IN MG. PER GM. OF DRY WEIGHT		NICOTINIC ACID LOSS
	Uncooked	Cooked	
			%
Chicken leg meat			
Frying	295	190	36
Frying	310	157	49
Frying	309	168	46
Frying	354	128	64
Roasting	297	189	37
Roasting	283	199	30
Steaming	320	175	45
Steaming	300	163	46
Chicken breast meat			
Frying	659	338	49
Frying	578	328	43
Frying	438	237	46
Frying	418	288	31
Roasting	585	392	33
Roasting	452	336	26
Steaming	629	416	34
Steaming	642	372	42
Beef liver			
Frying	370	222	40
Frying	314	250	20
Pork liver			
Frying	392	290	26
Frying	536	355	34
Pork liver (continued)			
Frying	454	366	19
Frying	450	345	23
Pork chops			
Frying	172	91	47
Frying	314	136	57
Frying	143	116	19
Frying	155	82	47
Frying	136	69	49
Ham			
Frying	143	131	8
Frying	128	89	30
Frying	89	76	15
Frying	91	72	21
Frying	170	132	22
Frying	139	111	20
Frying	139	109	22
Lamb heart			
Baking	298	123	59
Baking	270	138	49
Baking	293	132	55
Baking	301	98	67
Baking	264	114	57
Baking	279	131	53

We interpret these observations in the following manner. Agreement between the results of our own chemical method and the microbiological assay has been reported by Snell and Wright ('41) and recently demonstrated by Cheldelin ('42), for a number of foodstuffs analyzed by both procedures. It seems reasonable to assume that close correspondence between results obtained by two such widely different methods indicates that they lead to essentially true values for the nicotinic acid content. In that case the values obtained chemically by McIntire et al. ('41) are too high; and this is in agreement with our previous knowledge of chemical methods for determining nicotinic acid. The method they used was essentially that of Melnick and Field ('40), and earlier observations in this laboratory have shown that incomplete decolorization of the tissue digests by charcoal as used in this method leads to apparent nicotinic acid contents which are higher than the true values.

If this interpretation is correct, the dog bio-assay also gives results which are too high; possibly as has been suggested by Snell and Wright ('41) because the basal diet is incomplete apart from its deficiency of nicotinic acid and the meat supplements act in part by supplying other factors than nicotinic acid.

The results obtained by the bio-assay, being the first to appear, have been widely used in tables showing the nicotinic acid content of foodstuffs, but in the light of this interpretation it now appears that they are too high and that the later lower figures obtained on the same samples by the microbiological assay are more trustworthy.

It will be noticed that muscle meats for each mammalian species have been grouped together. This was done because no evidence of difference in nicotinic acid content of different muscle meats emerged from our figures. Thus the seventeen figures for beef muscle meats include round steak, 50, 51, 58, 58, and 56 μg. per gram; brisket, 58; chuck, 59, 71, and 40; neck, 31; rib, 82, 73, and 66; heart, 81; other cuts, 44, 55, and 44. Possibly rib steak may contain more nicotinic acid than

round steak, but more data would be required to establish this point.

Most of the types of meat show considerable variation from one sample to another. This is no doubt largely due to variations among individuals of the species but may also be partly due to effects of the ageing of the meat, a variable which we have not been able to control. Preliminary experiments with the tissues of rabbits have indicated a definite loss of nicotinic acid after storing the meat for 1 week in a household refrigerator. The possibility of destruction of nicotinic acid during the commercial ageing of meat of other species requires investigation.

The loss of nicotinic acid which we have observed during cooking is surprisingly large. This is at present inexplicable, but important in its application to dietetics. Since most meats are cooked before consumption, only one-half to two-thirds of the nicotinic acid present in the raw meat will generally survive cooking and be ingested; allowances for this must be made when calculating the nicotinic acid content of diets from data obtained by the analysis of raw meats.

Our figures show that the liver of the chicken takes its place with mammalian liver as one of the richest sources of nicotinic acid: it is equalled by the breast meat of the chicken, which is much richer than any other muscle meat we have examined.

SUMMARY

Nicotinic acid analyses of a number of meats purchased at retail stores are reported, based on a chemical procedure using completely decolorized digests of the tissue.

The values reported are considerably lower than have been obtained by others using either bio-assay on the dog or a chemical procedure with only partly decolorized digests, but they correspond more closely with results obtained in the microbiological assay and are believed to be more nearly correct.

The effect of cooking on several types of meat has been examined; generally from one-third to one-half of the nicotinic acid was lost, based on the dry weight of the meat.

Our thanks are due to the John and Mary R. Markle Foundation for the support of this work.

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CATARACT AND OTHER OCULAR CHANGES RESULTING FROM TRYPTOPHANE DEFICIENCY [1]

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In the past several years a series of investigations on nutritional cataracts have been made at this laboratory. It has been shown that one type of cataract and keratitis can be obtained readily in the eyes of rats fed riboflavin deficient diets (Day, Langston and O'Brien, '31; Day, Darby and Langston, '37). The discovery by Mitchell and coworkers that cataracts also resulted from feeding of excessive amounts of lactose (Mitchell and Dodge, '34) and galactose (Mitchell, '35) led to a study of other sugars as causative agents. It was reported from here (Darby and Day, '39) that xylose is likewise an effective agent for production of lens opacities. The xylose cataract, which in appearance is identical to that obtained by feeding galactose and lactose, is quite unlike riboflavin deficiency cataract.

The investigations to be reported in this communication were prompted by the observation of Berg and Potgieter [2] that many of their animals on tryptophane-deficient diets developed lens opacities. These workers were not satisfied

[1] Research paper no. 527, journal series, University of Arkansas. Most of the data contained in this report were presented at the 35th Annual Meeting of the American Society of Biological Chemists in Chicago, Illinois, April 18, 1941 (Totter and Day, '41). The authors are indebted to Miss Virginia Mims for technical assistance.

[2] Personal communication from Dr. C. P. Berg.

that a relationship between the amino acid deficiency and the cataract was sufficiently well-established and they made no comment upon it in their publication (Berg and Potgieter, '32).

A search of the literature revealed that Curtis, Hauge and Kraybill ('32) had recorded the occurrence of what they called "a type of permanent blindness" in rats maintained on a tryptophane-low diet.

EXPERIMENTAL

The compositions of the basic experimental diets are given in table 1. Diet 5000 was similar to the tryptophane-deficient

TABLE 1

Composition of the amino acid-deficient diets.

	DIET NO. 5000	5002
	%	%
Acid-hydrolyzed casein	14.7	
Cystine	0.3	
Whole zein		18.0
Sucrose	15.0	15.0
Starch	42.0	39.0
Agar	2.0	2.0
Salt mixture (Hubbell, Mendel & Wakeman, '37)	2.0	2.0
Cod liver oil	5.0	5.0
Hydrogenated cottonseed oil (Crisco)	19.0	19.0

B vitamins furnished by daily supplementation with 250 mg. yeast (Harris).

diet employed by Berg and Potgieter ('32). The acid-hydrolyzed casein was prepared by the method of Berg and Rose ('29). Whole zein [3], which is deficient in both tryptophane and lysine, was used as the chief source of dietary nitrogen in diet 5002.

Litters from two groups of rats were used, one a Wistar strain of albinos and the other a strain of black and white

[3] Purchased from the Prolamine Products Co. of Roby, Indiana, and used without further purification.

hooded animals. Young rats, immediately after weaning (except for two experiments in which older animals were used), were housed in individual wire-bottom cages and given water and the experimental diets ad libitum. Litter-mate controls received 0.1% or 0.2% tryptophane mixed with their diet. All animals were given the B vitamins by daily supplementation with 250 mg. of yeast [4]. Weight, food consumption and ophthalmoscopic records were made at weekly intervals. Slit-lamp examinations of the eyes of some of the animals were also made [5].

RESULTS AND DISCUSSION

The animals on the deficient diets lost weight rapidly and in a few days became emaciated and developed the characteristic hunchback and unkempt appearance typical of tryptophane deficiency in the rat.

Most of the small animals on diet 5000 eventually developed a severe alopecia, particularly on the face. However, the loss of hair was not shown by the animals on the zein diet, nor by the larger animals receiving diet 5000. It is not possible, from our data, to state definitely that the alopecia was due solely to a lack of tryptophane. Diet 5000 has a very oily consistency as contrasted to diet 5002 which is mealy, the zein being bulky enough to absorb much of the fat. Small animals could not easily be kept out of the food containers and the constant contact with the fat of the diet was very probably responsible for the greasy appearance of the hair and may have contributed to the development of the alopecia.

After several weeks on the deficient diets some animals exhibited a nervous manifestation characterized by hyperexcitability, especially to auditory stimuli.

The results of the ophthalmoscopic studies, summarized in table 2, indicate that the lenticular changes observed in the deficient animals are due solely to the lack of tryptophane in

[4] Harris.

[5] We are indebted to Dr. K. W. Cosgrove of the Department of Ophthalmology for the slit-lamp examinations.

TABLE 2

Incidence of cataract and keratitis in rats fed various amino acid deficient diets.

GROUP NO.	SUPPLEMENT	INITIAL BODY WEIGHT IN	NO. OF ANIMALS DEVELOPING			TIME IN DAYS FOR APPEARANCE OF		
			ophthalmoscopic cataract	gross cataract	keratitis	ophthalmoscopic cataract	gross cataract	keratitis
		gm.						
I (14)[1]	0.2% Tryptophane	30–48	0	0	0	....	....	....
II (40)	.....	30–50	37	8[2]	27	8–64	63–106	14–145
III (4)	120 μg. riboflavin per wk.	32–43	4	2	4	18	65	25–51
IV (6)[3]	0.2% Tryptophane	32–36	0	0	0	....	....	....
V (16)	0.1% or 0.2% Tryptophane	26–200	0	0	11	....	....	11–118
VI (14)	.....	26–44	12	2[4]	13	13–42	70–119	14–193
VII (6)	.....	58–78	5	1	5	40–82	86	11–112
VIII (4)	.....	130–210	0	0	3	....	....	25–46

[1] Groups I–IV, inclusive, were fed diet 5000; groups V–VIII, inclusive, received diet 5002. Numbers in parentheses indicate the total number of animals in the group.

[2] Only 21 animals were kept on the experimental diet longer than 63 days.

[3] The diet of these animals was restricted in amount to equal that eaten by deficient animals.

[4] Only 7 animals were kept on the experimental diet longer than 70 days.

their diet. The animals of groups I to IV received the hydrolyzed-casein diet. Those of group I received 0.2% tryptophane mixed with the diet and served as positive controls. All of the animals in this group grew at a good rate and none showed any of the deficiency signs exhibited by the animals of groups II and III. Group II received no tryptophane supplement, and thirty-seven of the forty animals developed ophthalmoscopic cataract in from 8 to 64 days, while twenty-seven also showed vascularity of the cornea. A few had a generalized ophthalmia. Of twenty-one animals in group II which were kept on the diet longer than 63 days, eight developed mature cataracts.

An assay for riboflavin in the yeast which was used as a source of B complex by the method of Darby and Day ('38) showed that it contained 45 μg. per gram. Therefore, the rats in this study each received 68 μg. weekly in the yeast supplement. Inasmuch as it was shown (Day, Darby and Langston, '37) that as little as 30 μg. of riboflavin weekly was adequate to protect against riboflavin deficiency cataract, it seems very unlikely that riboflavin deficiency was involved in the experiments here reported. However, to test this possibility additional amounts of riboflavin equal to 120 μg. weekly were given to the animals of group III. Such supplementation failed to alter significantly either the nature or the time of incidence of the ocular changes.

The results obtained with group IV offer additional proof that a vitamin lack was not responsible for the eye changes seen. The food intake of these animals, receiving diet 5000 supplemented with 0.2% tryptophane, was restricted to equal the intake of litter-mate controls receiving diet 5000 only. Hence, both experimental and control animals received the same quantity of all food factors except tryptophane. None of the group IV animals showed any of the pathological changes seen in groups II and III.

The animals in groups V to VIII received the zein diet which was, of course, deficient in lysine. Those of group V received 0.1% or 0.2% tryptophane mixed with their diet and served

as controls. None of them exhibited cataract but eleven out of sixteen did show vascularization of the cornea. The incidence of keratitis appeared to be the same at either level of tryptophane supplementation. Animals in group VI (deficient diet without supplement) exhibited both cataract and keratitis. Groups VII and VIII were larger animals and received the same diet as group VI. Both groups exhibited keratitis but the group containing the largest animals (VIII) did not show cataract before the termination of the experiment on the eighty-sixth day.

The eye changes seen were both lenticular and corneal, but the corneal manifestations, described below, appeared also in the eyes of many animals receiving a diet containing tryptophane but deficient in lysine. On the other hand, the lack of lysine does not appear to influence the lens changes. However, conclusions regarding specific lesions related to lysine deficiency should not be drawn from results obtained with our experimental regimen since the yeast vitamin supplement undoubtedly contains some of this amino acid. Very slow growth was obtained by use of diet 5002 supplemented with tryptophane, indicating that the amount of lysine received by the animals was not insignificant.

Changes in the lenses of animals on a tryptophane-deficient diet began to appear after 2 to 3 weeks on the experiment. The initial change was an apparent difference in optical density of lens tissue in the form of a concentric band or ring situated in the cortex of the lens. This usually progressed until there was a shell-like appearance of the lens cortex, visible entirely around the lens, both anteriorly and posteriorly. The diameter of this shell appeared to be approximately two-thirds the diameter of the lens. In about one-third of the animals remaining on the diet for a sufficiently long period the lenses became partially or entirely white and opaque. Frequently that part of the lens within the incipient "shell" became opaque, leaving clear lens fibers at the periphery. In other cases the entire lens became opaque. Such mature cataracts were plainly visible to the unaided eye.

The cataract produced by these experimental diets, as seen with the ophthalmoscope, was quite unlike lens opacities resulting from xylose or galactose feeding as well as those resulting from riboflavin deficiency.

Keratitis was observed in the eyes of most of the animals receiving either the tryptophane- or the lysine-deficient diet, as well as in the eyes of those on diets lacking in both amino acids. The most striking change seen was the marked vascularity. Vessels pushed anteriorly from the outer canthus and progressed towards the center until, in some cases, the entire cornea was eventually involved. This vascularity differs from that seen in riboflavin deficient animals in that the vessels in the latter case simultaneously invade the cornea from all parts of the periphery. Also unlike the vascularization resulting from riboflavin deficiency, which frequently occurs only after a diffuse corneal opacity becomes apparent, the vessels in this case usually invade a perfectly clear cornea.

SUMMARY

Young rats, from 26 to 78 gm. in weight, which were given a tryptophane-deficient diet developed cataractous changes in from 8 to 82 days. These changes were manifested with or without a concurrent lysine deficiency. The lenticular changes were prevented by supplementation of the diets with 0.1% or 0.2% tryptophane, either when the diet was restricted or given ad libitum. A large proportion of the animals which received diets deficient in both tryptophane and lysine or lacking in either of these amino acids developed vascularity of the cornea. A few showed a generalized ophthalmia.

With the aid of the ophthalmoscope the cataract seen in these animals could be readily distinguished from that resulting both from riboflavin deficiency and from the feeding of galactose or xylose. The vascularity of the cornea seen in these amino acid-deficient animals showed certain features which distinguished it from the vascularity of riboflavin deficiency.

In addition to the ocular lesions noted, other changes associated with the tryptophane deficiency were loss of weight, hunchback, unkempt appearance, alopecia, greasy hair, and nervousness.

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NICOTINIC ACID, PANTOTHENIC ACID AND PYRIDOXINE IN WHEAT AND WHEAT PRODUCTS [1]

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Since wheat is an important constituent of the average American diet, it is obviously desirable to extend our knowledge about the vitamin content of this cereal. The flour enrichment program has increased the interest in this problem. Booth ('40) has reported on the thiamine content of wheats grown in various parts of the world. Schultz, Atkin and Frey ('39, '41) have made a survey of the vitamin B_1 content of American cereals. Nordgren and Andrews ('41) have studied the vitamin B_1 content of American and Canadian wheats, while Downs and Cathcart ('41) have made a study of the thiamine content of commercial wheats of the 1940 crop. Andrews, Boyd and Terry ('42) and Conner and Straub ('41 a, '41 b) have recently studied the distribution of riboflavin in cereals. Taylor ('41), Tobey and Cathcart ('41) and Tisdall et al. ('41) have discussed problems concerned with the fortification program, and have presented data on the distribution of thiamine and riboflavin in wheat and wheat products. Bin-

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The samples used in this study were obtained through the courtesy of the following: Minneapolis Federal Grain Inspection; Dr. H. G. L. Strange, Searle Grain Company, Ltd., Winnipeg, Canada; Mr. O. E. Barbee, State College of Washington, Pullman, Washington; Dr. John H. Parker, Kansas Wheat Improvement Assoc., Manhattan, Kansas; and Dr. C. A. Lamb, Ohio Agric. Exper. Station, Wooster, Ohio.

nington and Andrews ('41) have recently reported on the distribution of vitamin E in products of cereal milling.

As far as we know, no systematic study of the distribution of other vitamins in wheat has been reported. In our investigation, a number of varieties of wheat grown in various parts of the United States and Canada have been assayed for their nicotinic acid, pantothenic acid, and pyridoxine content.

EXPERIMENTAL

The nicotinic acid assays were made by the microbiological method of Snell and Wright ('41). This method employs the response of Lactobacillus arabinosus 17-5 to nicotinic acid or nicotinamide. The response is measured by titrating the acid produced. Samples were prepared for analysis as follows: 0.2 gm. of finely-ground wheat was suspended in 75 cc. of H_2O, 20 cc. of 8% NaOH were added, and the suspension was autoclaved for 20 minutes at 15 pounds pressure. It was then neutralized with HCl and diluted to 200 cc. Treatment of the sample on the steam bath for 1 hour with 5 N HCl gave identical results. Recovery of added nicotinic acid ranged from 90% to 110%.

It has been found by various workers that the microbiological assay of a water suspension of wheat for nicotinic acid gives values approximately 20% lower than the assay of a NaOH treated suspension. The effect is apparently not one of increased extraction, since a water filtrate is similarly increased in potency on treatment with NaOH. It would seem that the increase is due to the freeing of nicotinic acid from an inactive compound. This inactive compound is extremely labile to alkali since ¼% NaOH at room temperature gives the full effect within 5 minutes. Treatment with moderately strong acid or prolonged heating at neutrality will also bring about the conversion. Studies are now in progress to determine whether the increased activity, as measured by the microorganisms (after alkali treatment), is due to a compound biologically active in the animal body.

Pantothenic acid was determined by the microbiological method of Strong, Feeney and Earle ('41). In this method the response of Lactobacillus casei ε to pantothenic acid is measured by titrating the acid produced. Preparation of the sample was carried out as follows: 0.2 gm. of finely-ground wheat was suspended in 100 cc. of H_2O, autoclaved for 20 minutes at 15 pounds pressure, and diluted to 500 cc. Recovery of added pantothenic acid ranged from 90% to 110%. Strong, Feeney and Earle have found that treatment of cereals with enzymes does not produce a significant change in apparent pantothenic acid as measured by the microbiological method. Values are expressed in micrograms of calcium pantothenate per gram.

Pyridoxine was determined by the biological method of Conger and Elvehjem ('41). Twenty-one-day-old rats were depleted for 2 weeks on a pyridoxine low ration and the ration was then supplemented with the sample. Crystalline pyridoxine hydrochloride was fed at levels of 50, 75 and 100 μg. per 100 gm. of ration. The pyridoxine content of the sample was determined by interpolation on the standard curve obtained; values are given in micrograms of vitamin $B_6 \cdot HCl$ per gram.

In order to guard against group variation due to errors in assay procedure, representative samples from the various groups were assayed at the same time. All values are expressed as micrograms per gram air dry sample.

RESULTS AND DISCUSSION

Table 1 gives the results of analyses of four varieties of dark hard winter wheat grown at four different localities in Kansas and Nebraska. The Chiefkan variety samples from all locations and each of the varieties grown at Hutchinson contain slightly more nicotinic acid than the other samples. In the case of pantothenic acid and pyridoxine, neither varietal nor environmental differences seem to have any significant effect on the amount present. However, the Blackhull variety

TABLE 1

Nicotinic acid, pantothenic acid and pyridoxine content of dark hard winter wheats.

VARIETY	LOCATION GROWN				
	LINCOLN	DODGE CITY	WICHITA	HUTCHINSON	AVERAGE
	μg./gm.	*μg./gm.*	*μg./gm.*	*μg./gm.*	*μg./gm.*
			Nicotinic acid		
Chiefkan	61	67	66	66	66
Blackhull	57	60	53	59	59
Turkey	53	54	61	58	58
Tenmarq	56	54	56	57	57
Average	57	59	59	60	60
			Pantothenic acid		
Chiefkan	13.7	14.9	15.3	14.4	14.6
Blackhull	13.1	15.3	13.1	15.8	14.3
Turkey	14.1	15.6	16.0	15.5	15.3
Tenmarq	15.2	15.4	15.5	17.5	15.9
Average	14.0	15.3	15.2	15.8	15.0
			Pyridoxine		
Chiefkan	4.4	4.1	5.2	4.3	4.5
Blackhull	4.0	4.2	3.7	3.5	3.8
Turkey	5.4	4.6	5.8	5.7	5.4
Tenmarq	5.4	6.1	4.1	4.4	5.0
Average	4.8	4.8	4.7	4.5	4.7

samples contain appreciably lower amounts of pyridoxine than the other varieties.

In table 2 the values for miscellaneous dark hard winter wheats grown in Kansas, Minnesota, Oklahoma, Texas and Washington are given. The range of concentration of the three vitamins in these samples is approximately the same as in the samples obtained from Kansas and Nebraska listed in table 1, but the values tend to be lower. None of the dark hard winter wheats listed in table 2 contains as much nicotinic acid as the average of the samples reported in table 1. There is considerable overlapping in the case of pantothenic acid and pyridoxine in this group, but the average values in table 2 are appreciably lower than those in table 1 as may be seen from the data given in table 3. Table 2 also gives the values

TABLE 2

Nicotinic acid, pantothenic acid and pyridoxine content of miscellaneous wheats.

VARIETY	SOURCE	NICOTINIC ACID	PANTOTHENIC ACID	PYRIDOXINE
		μg./gm.	μg./gm.	μg./gm.
Dark Hard Winter Wheats:				
Turkey	Manhattan, Kansas	50	14.4	3.7
Turkey	Meno, Oklahoma	55	16.0	4.1
Turkey	Pullman, Washington	51	9.1	3.2
Tenmarq	Orienta, Oklahoma	52	12.4	3.5
Chiefkan	Guthrie, Oklahoma	51	11.8	4.7
Blackhull	Eagle City, Oklahoma	50	13.1	4.1
Blackhull	Claude, Texas	53	12.6	5.6
Minturki	Minneapolis, Minnesota	51	14.5	3.2
Montana	Minneapolis, Minnesota	47	11.9	3.7
Kharkof	Pullman, Washington	58	12.7	5.4
Soft Red Winter Wheats:				
Kawvale	Manhattan, Kansas	52	14.4	4.6
Kawvale	Lincoln, Nebraska	59	15.7	6.3
Kawvale	Talala, Oklahoma	55	15.1	3.9
Fulcaster	Wooster, Ohio	58	10.4	4.2
Wabash	Wooster, Ohio	52	9.3	4.9
Red Rock	Wooster, Ohio	62	9.4	4.7
Fultz	Wooster, Ohio	67	10.6	4.8
Thorne	Wooster, Ohio	58	9.4	4.6
Purdue	Wooster, Ohio	61	9.4	4.6
Soft White Winter Wheats:				
American Banner	Wooster, Ohio	62	11.4	4.7
Yorkwin	Wooster, Ohio	59	10.9	5.0
Dawson	Wooster, Ohio	65	10.9	4.3
White Club Wheat:				
Hymar	Pullman, Washington	56	11.4	4.5
Dark Northern Spring Wheats:				
Ceres	Minneapolis, Minnesota	52	15.0	3.6
Marquis	Minneapolis, Minnesota	48	15.3	3.8
Thatcher	Minneapolis, Minnesota	67	17.0	4.2
Nordhaugen	Minneapolis, Minnesota	53	13.9	4.0
Red Bobs	Winnepeg, Manitoba	76	14.7	5.6
Thatcher	Winnepeg, Manitoba	106	13.3	5.1
Garnet	Winnepeg, Manitoba	59	11.0	5.1
Reward	Winnepeg, Manitoba	64	12.5	5.1
Renown	Winnepeg, Manitoba	70	14.7	4.8
Garnet	LaCombe, Alta.	55	9.8	3.8
Thatcher	Saltcoats, Sask.	77	14.0	4.6
Hard White Spring Wheats:				
Baart No. 38	Pullman, Washington	53	11.3	4.8
Federation	Pullman, Washington	57	11.4	4.4
Blue Stem	Pullman, Washington	56	15.2	4.7
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Blackhull	Claude, Texas	53	12.6	5.6
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White Club Wheat:				
Hymar	Pullman, Washington	56	11.4	4.5
Dark Northern Spring Wheats:				
Ceres	Minneapolis, Minnesota	52	15.0	3.6
Marquis	Minneapolis, Minnesota	48	15.3	3.8
Thatcher	Minneapolis, Minnesota	67	17.0	4.2
Nordhaugen	Minneapolis, Minnesota	53	13.9	4.0
Red Bobs	Winnepeg, Manitoba	76	14.7	5.6
Thatcher	Winnepeg, Manitoba	106	13.3	5.1
Garnet	Winnepeg, Manitoba	59	11.0	5.1
Reward	Winnepeg, Manitoba	64	12.5	5.1
Renown	Winnepeg, Manitoba	70	14.7	4.8
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Burbank	Minneapolis, Minnesota	53	15.0	5.5
Soft White Spring Wheat:				
Dicklow	Ogden, Utah	56	11.9	4.9

obtained on miscellaneous soft winter wheats. The nicotinic acid and pyridoxine contents of these samples fall in the regular range of the other samples studied, while the pantothenic acid content of all the wheats obtained from Wooster, Ohio, is comparatively low (table 3). These results indicate that genetic differences are of little consequence in determining the pantothenic acid content of soft winter wheat, while environmental differences have considerable effect. The values obtained on miscellaneous spring wheats are also shown in

TABLE 3

Range of values for contents of nicotinic acid, pantothenic acid and pyridoxine.

SOURCE	NUMBER OF SAMPLES	NICOTINIC ACID		PANTOTHENIC ACID		PYRIDOXINE	
		Ave.	Range	Ave.	Range	Ave.	Range
		μg./gm.		*μg./gm.*		*μg./gm.*	
Kansas and Nebraska	16	60	53–71	15.0	13.1–17.5	4.7	3.5–6.1
Kansas, Minnesota, Oklahoma, Texas and Washington	10	52	47–58	12.8	9.1–16.0	4.1	3.2–5.6
Wooster, Ohio	9			10.2	9.3–11.4		
Kansas, Nebraska, Oklahoma and Washington	4			14.1	11.1–16.7		
Canada	7	74	55–106				
Minnesota, Utah and Washington	9	53	48–67				

table 2. Samples obtained from Canada are definitely higher in nicotinic acid content (table 3). No corresponding variation was found in the case of pantothenic acid and pyridoxine. The hard white spring wheats from Minnesota and Washington listed in table 2 show a vitamin content that falls in the range of the other samples studied.

More than fifty samples of commercially milled patent and clear flours have been assayed for nicotinic acid. Three samples giving typical values have also been assayed for pantothenic acid and pyridoxine. The results, which are collected in table 4, indicate that patent flour, compared with whole wheat, contains approximately one-sixth as much nicotinic acid, and

about half as much pantothenic acid and pyridoxine. It has been shown that in the milling of patent flour, thiamine is left behind in the coarser fractions in proportions comparable to the nicotinic acid. If one wishes to restore the vitamin balance of whole wheat to patent flour, it is of major importance to add thiamine and nicotinic acid.

It is interesting to note that although there is a concentration of thiamine, riboflavin, and pyridoxine in the germ of the wheat kernel, wheat germ is about equal to whole wheat in pantothenic acid and tends to be lower than whole wheat in nicotinic acid. The use of small amounts of wheat germ in flour, which some have advocated as a measure to give flour

TABLE 4

Nicotinic acid, pantothenic acid and pyridoxine in wheat flours and wheat germ.

SAMPLE	NICOTINIC ACID	LOSS IN MILLING	PANTOTHENIC ACID	LOSS IN MILLING	PYRIDOXINE	LOSS IN MILLING
	μg./gm.	%	*μg./gm.*	%	*μg./gm.*	%
Average value for 55 samples of whole wheat	59		13.3		4.6	
Patent flour	10	83	5.7	57	2.2	52
First clear flour	21	64	9.6	28	3.9	15
Second clear flour	57	3	12.8	4	5.7	
Wheat germ	34		15.3		9.6	

more nearly the nutritive properties of whole wheat, means a contribution of much less pantothenic acid and nicotinic acid than vitamin B_1, riboflavin, or vitamin B_6. It would appear that the great improvement which is imparted by using 5% of germ in bread (such as reported by McHenry, '40) cannot be attributed to the addition of these two members of the B-complex.

SUMMARY

Fifty-five samples of wheat, varying as to variety and source, have been assayed for nicotinic acid, pantothenic acid, and pyridoxine. Patent flour, clear flours, and wheat germ have also been assayed for these vitamins. Patent flour, compared

with whole wheat, was found to contain about one-sixth as much nicotinic acid, and approximately one-half as much pantothenic acid and pyridoxine.

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THE RELATION OF B-VITAMINS AND DIETARY FAT TO THE LIPOTROPIC ACTION OF CHOLINE[1]

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Junkersdorf and Kohl ('26) observed that choline lowered liver-fat levels in dogs. A similar choline effect was demonstrated in rats by Best, Hershey and Huntsman ('32), and by a number of workers since that time. Studies concerning the relation of the B-vitamins to liver-fat metabolism originated when Whipple and Church ('36) demonstrated that thiamine increases liver-fat in the rat. These results were confirmed by McHenry ('37). Gavin and McHenry ('40) reported that the thiamine-induced fatty liver was not affected by pyridoxine, nicotinic acid, or riboflavin, but that choline administration restored liver-fat to normal levels. Engel and Phillips ('39) observed increases in liver-fat in thiamine-deficient rats which were fed thiamine, but they were unable to prevent this condition with choline. This lack of agreement suggests that other B-vitamins might be concerned with the metabolism of liver-fat, since the diet employed by the latter workers contained a B-vitamin concentrate (liver-filtrate factor).

McHenry and Gavin ('40) found that a beef liver fraction containing pantothenic acid and "factor W" produced a marked increase in liver-fat which could be prevented by "lipocaic," a pancreatic extract. More recently Gavin and McHenry ('41) have shown that a similar fatty liver could be produced by biotin and prevented by inositol.

[1] Published with the approval of the Director of the Alabama Agricultural Experiment Station. A preliminary report of this work was presented before the 35th meeting of the American Society of Biological Chemists at Chicago, Illinois (J. Biol. Chem., vol. 140, p. xxxvii, 1941).

Halliday ('38) reported that rats on pyridoxine-deficient diets had fatty livers which could not be entirely prevented by choline. Gavin and McHenry ('40) were unable to confirm these results.

The above citations strongly indicate that a number of B-vitamins are concerned with the metabolism of liver fat. In most of the work cited, these vitamin effects were produced in animals receiving diets which were high in fats or low in casein. Casein is known to have a lipotropic action (Channon and Wilkinson, '35; Beeston et al., '35). It seemed desirable, therefore, to determine whether liver-fat metabolism could be influenced by the addition of accessory food factors to a diet containing 18% of casein. Such diets are in common use in nutrition studies. Furthermore, it was of interest to investigate the lipotropic action of choline under such dietary conditions and to determine if normal levels of liver-fat could be maintained in growing rats receiving a purified diet supplemented with crystalline vitamins.

MATERIALS AND METHODS

The basal diet used in these studies consisted of casein [2] 18, sucrose 78, and salt mixture [3] 4%. The diet was supplemented once weekly with adequate amounts of carotene and calciferol. The remaining accessory food factors were supplied daily, mixed with a small portion of the basal diet. As a routine procedure, litters of eight rats were divided into two like groups as to number, weight, and sex. By such means it was possible to study the effect of a given vitamin within a litter.

The rats were weaned at 23 days of age, placed in individual cages with raised screen floors, and, unless otherwise indicated, were fed the experimental diet for a period of 3 weeks. At the end of this period the animals were killed by carotid bleeding. The livers were weighed, dried at 104°C., reweighed, ground, and extracted with dry ethyl ether for 16 hours. The liver-fat values reported represent the per cent ether extract

[2] Labco, from Borden Company.
[3] J. Biol. Chem., vol. 89, p. 199, 1930.

on a dry weight basis. In order to determine normal values for liver-fat in rats of this age, livers from twenty-four animals which had been fed the stock diet for 3 weeks were treated in the manner described above. Data thus obtained showed that the average liver-fat in such animals was 5.25 ± 0.20%.

RESULTS

The relation of thiamine, riboflavin, pyridoxine, and pantothenic acid to the lipotropic action of choline. The relation of thiamine, riboflavin, pyridoxine, and pantothenic acid to the metabolism of choline was studied by omitting them singly from the diet and comparing the data thus obtained with data obtained when all these factors were present in the diet. The various levels at which choline was fed, and the daily supplements of the other accessory factors are given in the accompanying tables. The results of this series of experiments are presented in table 1.

When thiamine, riboflavin, or pantothenic acid was omitted from the diet, approximately normal values for liver-fat were obtained even though the daily choline chloride intake was only 2 mg. The need of additional dietary choline as a lipotropic agent was apparent when thiamine, riboflavin, and pantothenic acid were all present in the diet. The increased need for choline was correlated with increased food consumption and with increased body weight. The omission of pyridoxine from the diet for a 3-week experimental period had no effect on liver-fat values.

That the abnormal liver-fat deposition in the rats receiving both low levels of choline and all the B-vitamins was mainly caused by a lack of dietary choline is demonstrated by the reduction in liver-fat which occurred with increased choline feeding. At the 2 mg. level of choline chloride, hemorrhagic kidneys were frequently encountered, and in a few instances death occurred from choline deficiency. When the choline chloride intake was raised from 2 mg. to 5 mg. per rat daily, no hemorrhagic kidneys were observed. This increase in dietary choline had a marked lipotropic effect and also pro-

TABLE 1

Average growth, food consumption, and percentage liver fat under varying B-vitamin and choline intake.

GROUP [1]	DAILY CHOLINE CHLORIDE INTAKE	THIAMINE			RIBOFLAVIN			PANTOTHENIC ACID			PYRIDOXINE		
		Body weight [2]	Daily food intake	Liver fat	Body weight [2]	Daily food intake	Liver fat	Body weight [2]	Daily food intake	Liver fat	Body weight [2]	Daily food intake	Liver fat
	mg.	*gm.*	*gm.*	%	*gm.*	*gm.*	%	*gm.*	*gm.*	%	*gm.*	*gm.*	%
A	2	39 71	...	33.10	38 62	...	38.50	45 75	...	26.85	40 72	...	28.85
B	2	42 33	...	5.10	37 41	...	9.25	42 56	...	9.90	44 59	...	21.95
A	5	45 90	6.1	18.50	42 84	5.0	12.80	47 90	5.8	17.90	42 78	5.4	18.60
B	5	46 50	3.2	6.30	41 52	3.0	7.65	47 79	5.0	14.30	43 65	4.1	18.20
A	10	54 112	7.7	12.40	42 88	6.0	10.15	39 82	...	12.60	42 88	6.0	11.75
B	10	53 53	3.0	5.00	44 48	3.1	3.90	40 78	...	6.00	42 66	4.1	11.15
A	20	56 108	6.2	10.15	55 96	5.0	10.20	50 88	6.1	7.95	55 96	5.1	7.15
B	20	55 60	3.4	5.60	54 60	3.6	3.60	48 78	5.4	6.00	54 82	3.8	6.40
A	40	57 106	...	10.70	45 75	4.7	8.70	57 79	...	7.00	43 91	...	13.85
B	40	55 50	...	5.05	44 49	3.0	7.85	55 75	...	4.90	42 64	...	10.15
A	70	49 87	5.2	11.20	48 79	6.3	9.05	44 78	4.7	7.40	39 87	4.5	7.60
B	70	49 44	2.8	5.20	48 55	3.5	7.45	46 76	4.2	4.25	40 65	3.5	11.65

[1] Each rat in group A received daily 20 μg. of thiamine, riboflavin, and pyridoxine; 100 μg. of calcium pantothenate, and 0.1 ml. of corn oil. The animals in group B received the same supplements with B-vitamins omitted, one at a time, as indicated by the table headings. Each experimental result represents an average obtained from eight rats.

[2] The first column of figures represents initial body weight, the second column represents final body weight.

duced a definite growth response. Although the results were somewhat variable, in general it would appear that, under these dietary conditions, 10 mg. of choline chloride per rat daily produced the maximum lipotropic effect. Feeding amounts larger than 10 mg. failed to lower the liver-fat values to any significant degree, and there was some indication that the higher levels (40 to 70 mg.) resulted in a slight growth inhibition.

Dietary choline, even at high levels, failed to restore liver-fat values to normal if the diet contained thiamine, riboflavin, calcium pantothenate, and pyridoxine. The absence of other lipotropic agents from the diet seemed a logical explanation. Since inositol has been demonstrated to have a lipotropic action under certain dietary conditions (Gavin et al., '41) the action of this substance was determined in the following series of experiments.

The relation of inositol to the lipotropic action of choline. To determine the action of inositol, rats were fed the basal diet plus thiamine, riboflavin, pyridoxine, pantothenic acid and inositol as the B-vitamin supplements. Litter-mate rats receiving the same diet and supplements, with the exception of inositol, served as controls. Choline was again fed to both groups at varying levels. The amounts of the various supplements fed are presented with the results in table 2.

As in the previous experiments, the animals receiving thiamine, riboflavin, pyridoxine, pantothenic acid, and adequate choline had liver-fat values appreciably above normal. With the addition of inositol, however, the liver-fat was reduced to the levels found in rats receiving a stock diet. The results show clearly that both choline and inositol are necessary as lipotropic agents for rats receiving purified diets containing thiamine, riboflavin, pyridoxine, and pantothenic acid.

The relation of pyridoxine to the lipotropic action of choline as influenced by the duration of the experiment. In the previous experiments it was apparent that pyridoxine had no effect on liver-fat levels if the test feeding period was limited to

TABLE 2

Average growth, food consumption, and percentage liver fat with and without inositol in the diet, and with varying levels of choline intake.

GROUP [1]	DAILY CHOLINE CHLORIDE INTAKE	BODY WEIGHT		DAILY FOOD INTAKE	LIVER FAT
		Initial	Final		
	mg.	*gm.*	*gm.*	*gm.*	%
A	5	46	92	6.0	20.25
B	5	46	94	6.2	14.10
A	10	47	95	6.0	8.80
B	10	48	96	6.0	6.35
A	20	44	88	5.8	8.70
B	20	45	88	5.6	5.75
A	40	48	92	4.9	6.50
B	40	48	90	4.8	5.10

[1] Each rat in group A received daily, 20 μg. each of thiamine, riboflavin, and pyridoxine, 100 μg. of calcium pantothenate, and 0.1 ml. of corn oil. Those in group B received the same supplement plus 3 mg. of inositol per rat daily. Each experimental result represents an average obtained from twelve rats.

3 weeks. Since it has been reported (Halliday, '38) that rats deficient in pyridoxine have fatty livers, it seemed desirable to determine the influence of this factor on the lipotropic action of choline under more severe deficiency conditions. In these experiments each rat received 10 mg. of choline chloride daily. The vitamin supplements which the rats received daily over varying experimental periods are presented with the results in table 3.

After a 3-week feeding period the pyridoxine-deficient animals had liver-fat values comparable to those of the control group receiving pyridoxine. Similar results were obtained after a 6-week period. By the end of the eighth week, however, the pyridoxine-deficient animals had accumulated abnormal quantities of liver fat. These abnormal liver-fat levels were also present in the rats receiving the pyridoxine-deficient diet for 14 and 24 weeks, respectively.

On the basis of these results it would seem that choline was less effective as a lipotropic agent in rats receiving a diet deficient in pyridoxine than in rats receiving the same diet

supplemented with pyridoxine. The results indicate that the animals receiving pyridoxine in this series of experiments had liver-fat values somewhat higher than those reported for rats receiving the stock diet. On the basis of the results with inositol in the preceding experiments it would seem that this abnormality was due to a deficiency of inositol. Experiments are in progress to clarify this point.

TABLE 3

The effects on liver-fat levels of essential fatty acids (corn oil) and pyridoxine in the diet.

DIET WITH AND WITHOUT PYRIDOXINE					DIET WITH AND WITHOUT ESSENTIAL FATTY ACIDS (CORN OIL)				
Group [1]	Period	Average body weight		Average liver fat	Group [2]	Period	Average body weight		Average liver fat
		Initial	Final				Initial	Final	
	weeks	*gm.*	*gm.*	*%*		*weeks*	*gm.*	*gm.*	*%*
A	3	41	82	11.2	A	3	42	89	11.4
B	3	42	96	11.8	B	3	41	94	11.7
A	6	45	85	12.6	A	8	44	120	25.0
B	6	43	135	13.2	B	8	46	166	11.5
A	8	45	93	22.5	A	10	48	124	25.8
B	8	43	143	11.4	B	10	50	199	13.5
A	14	44	85	16.8	A	12	49	168	18.3
B	14	45	190	9.5	B	12	48	244	8.9
A	24	44	86	38.5	A	20	44	197	20.8
B	24	45	221	17.6	B	20	44	290	15.2

[1] Each rat in group A received daily 20 μg. each of thiamine and riboflavin, 100 μg. of calcium pantothenate, 0.1 ml. of corn oil, and 10 mg. of choline chloride. Those of group B received the same supplement plus 20 μg. of pyridoxine per rat daily. Each experimental result represents an average obtained from four rats.

[2] Each rat in group A received daily 10 μg. each of thiamine and pyridoxine, 0.5 gm. of Lilly's liver extract 343, and 10 mg. of choline chloride. Those in group B received the same supplement plus 0.1 ml. of corn oil (Mazola) per rat daily. Each experimental result represents an average obtained from four rats.

The relation of essential fatty acids (corn oil) to the lipotropic action of choline as influenced by the duration of the experiment. In the previous experiments corn oil was included in the diet as a source of essential fatty acids. Preliminary trials had indicated that this dietary supplement had no in-

fluence on the lipotropic action of choline over a 3-week experimental period. Since other investigators have reported that essential fatty acids and pyridoxine mutually function in curing rat acrodynia, and since pyridoxine influenced the lipotropic action of choline in experiments of longer duration, it seemed desirable to conduct a series of experiments with, and without, corn oil in the diet. For these experiments the casein of the basal diet was subjected to four 2-hour extractions with boiling 95% ethyl alcohol. The accessory food factors fed daily are shown with the results in table 3. Choline chloride was again fed at a level of 10 mg. per rat daily.

The liver-fat levels in the rats receiving corn oil showed no significant changes regardless of the duration of the experiment. After an 8-week feeding period the liver-fat in the rats receiving the fat-free diet had increased approximately 100%. Similar increases were observed if the experimental period was extended to 10, 12, or 20 weeks.

These results indicate that the 10 mg. daily choline intake was less effective as a lipotropic agent in the fat-deficient animal than it was in the animals receiving a source of essential fatty acids. Thus it would seem that pyridoxine and essential fatty acids act mutually in influencing the lipotropic action of choline and are complementary in this respect.

DISCUSSION

The basal diet used in these studies is comparable to those used by many investigators concerned with the nutrition of the various B-vitamins. The results reported herein indicate that the abnormal liver-fat deposition resulting from the feeding of thiamine, riboflavin, and pantothenic acid should not be considered as a harmful effect of these vitamins. This abnormality was entirely alleviated by fortifying the diet with adequate amounts of the known lipotropic agents, choline and inositol, and must be regarded therefore as representing a deficiency of these factors. Both choline and inositol are

essential, in a purified diet containing 18% casein and supplemented with crystalline vitamins, if normal levels of liver-fat are to be maintained.

It is difficult to compare the choline requirement as found in the present study with those reported by other investigators, because of variations in the diets. Griffith ('41) has reported that, for diets containing 18 to 24% of casein and 6% of yeast, 4 to 6 mg. of choline chloride was needed daily to prevent the accumulation of liver lipids. Data herein presented indicate that at least 10 mg. of choline chloride per rat daily is necessary for the maintenance of normal liver-fat levels. Work now in progress on chemical and biological assays of various yeasts for their choline content indicates that the choline requirement suggested by Griffith is in good agreement with that found in the present investigation, if the choline supplied by the yeast is taken into account.

That pyridoxine and corn oil decrease the deposition of liver fat is of interest in view of the reports that both these factors are concerned in the cure of rat acrodynia (Birch, '38; Salmon, '38, '40). The observation that fatty livers result if rats are deprived of pyridoxine over long periods of time is a confirmation of results reported by Halliday ('38). Gavin and McHenry ('40) failed to observe any effect of pyridoxine on liver-fat if rats were fed first a depletion diet for 3 weeks and then various test supplements including pyridoxine for 1 week. Present results indicate that an 8-week depletion period is necessary before a pyridoxine effect can be demonstrated.

It is of interest to note that inositol and choline are both lipotropic agents and that both have been reported to have a protective action against perosis in chicks. Wiese and associates ('38) reported that inositol afforded protection against perosis in chicks receiving insufficient manganese. Hegsted and associates ('41) and Hogan and associates ('41) have reported that choline prevents perosis.

SUMMARY AND CONCLUSIONS

When thiamine, riboflavin, pantothenic acid, pyridoxine, corn oil, and choline were fed to rats receiving a purified diet containing 18% of casein for a 3-week experimental period, an abnormal accumulation of liver-fat resulted. Under these conditions 2 mg. of choline chloride per rat daily failed to prevent the kidney hemorrhages of choline deficiency; at least 10 mg. of choline chloride was necessary for this factor to exert its maximum lipotropic action but normal liver-fat levels were still not obtained.

The addition of 3 mg. of inositol per rat daily to the diet adequate in choline and containing the above B-vitamins reduced the liver fat to the normal level found in rats receiving an adequate stock diet.

Prolonged feeding of a diet deficient in pyridoxine or essential fatty acids resulted in fatty livers, even though the diet contained adequate choline.

It is concluded that pyridoxine and a source of essential fatty acids are necessary in the diet for choline to function properly as a lipotropic agent. Inositol, in addition to choline, is a necessary dietary constituent for the rat receiving purified diets supplemented with the other B-vitamins known to be required by this species.

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FACTORS REQUIRED BY CHICKS MAINTAINED ON A HEATED DIET [1]

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In 1932 a heated grain and casein diet was first used in our laboratory for nutritional studies with chicks (Kline, Keenan, Elvehjem and Hart, '32). This ration was found to be useful in studies dealing with the identification of pantothenic acid as the chick antidermatitis factor (Woolley, Waisman and Elvehjem, '39) and for the assay of pantothenic acid in food materials (Waisman, Mickelsen and Elvehjem, '39). While this ration was quite satisfactory for such studies, it soon became apparent that factors other than pantothenic acid were necessary to render the diet complete. We wish to report here the results of several years of work on the heated diet and the data that have been obtained on further factors required by the growing chick.

EXPERIMENTAL

The basal diet used throughout was a modification of the original diet described by Kline, Keenan, Elvehjem and Hart ('32). It consists (in per. cent) of ground yellow corn 56, standard wheat middlings 25, crude casein 12, soy bean oil 3, and a salt mixture 4. The chicks were given 2 drops halibut liver oil weekly to insure the supply of vitamins A and D. Although the original workers resorted to 100-hour

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. We are greatly indebted to Wilson Laboratories for the various liver fractions, to Abbott Laboratories for halibut liver oil, and to Merck and Company for the synthetic vitamins used throughout these experiments.

heating of the grains and casein part of the diet at 100°C., later work (Mickelsen, Waisman and Elvehjem, '38) showed that a 30-hour treatment of the diet at 120°C. gave more consistent dermatitis.

In the majority of our experiments 100 gm. of the basal ration were supplemented with 300 μg. thiamine, 400 μg. riboflavin, 400 μg. pyridoxine, 100 mg. choline, and 0.50 mg. of 2-methyl,-1,4-naphthoquinone. There was no growth stimulation when all of these vitamins were included in the basal ration, but the chicks did show greater survival time.

Pantothenic acid requirement

The availability of crystalline calcium pantothenate enabled us to study both the level necessary to completely protect the birds from the typical dermatitis and the level required to give maximum growth. A large number of groups of day-old White Leghorn chicks were fed the basal diet (with the vitamins mentioned above) plus levels of calcium pantothenate ranging from 100 μg. to 2000 μg. per 100 gm. of diet. Typical weights of the birds are given in table 1. Complete protection from all symptoms was obtained with 300 μg. of calcium pantothenate per 100 gm. of diet. Below this figure there were cases of the typical incrustations.

There was observed a corresponding growth increase with each increment of the vitamin up to 1000 μg. per 100 gm. of diet. In several groups a 750 μg. level gave nearly maximum growth and only one or two times did the average weight of the chicks on the 1000 μg. level exceed the growth obtained on the 750 μg. level. The variation is thought to be due to storage of other essential factors in different batches of chicks. The maximum growth obtained on 750 μg. is in contrast to that reported by Jukes ('39) who found 1400 μg. to give optimum growth on a similar ration. It should be pointed out, however, that Jukes depleted the chicks for a short period before the pantothenic acid was given, while our chicks received the pantothenate from the start. The data do show that the

amount of vitamin needed for prevention of the dermatitis is less than that necessary to promote maximum growth.

It was apparent from these experiments that the chicks receiving the heated diet plus adequate amounts of calcium pantothenate did not give the growth considered to be optimum for White Leghorn chicks. The optimum weight for this breed in our laboratory has been found to be about 230 gm. at 4 weeks of age. In an effort to attain optimum growth in the chicks fed the heated diet, experiments were devised to study the effect of several food factors which had been proposed for the chick, but which had not been chemically defined. To this

TABLE 1

Typical growth responses of chicks maintained on the heated diet to increasing levels of calcium pantothenate.

	WEIGHT OF BIRDS AT 4 WEEKS	PANTOTHENIC ACID DERMATITIS
	gm.	
241H basal	65	++++
+ 100 μg. P.A.[1]/100 gm.	85	+++
+ 200 μg. P.A./100 gm.	98	++
+ 300 μg. P.A./100 gm.	111	—
+ 500 μg. P.A./100 gm.	122	—
+ 750 μg. P.A./100 gm.	132	—
+ 1000 μg. P.A./100 gm.	148	—
+ 15 mg. P.A./kg.	133	—

[1] Calcium pantothenate.

end a variety of liver fractions, yeast preparations and other sources of vitamins were added to the basal ration plus adequate amounts of calcium pantothenate. Because of the large amount of data gathered during our extended experiments, the material will be presented in highly condensed form with a short discussion of the individual fractions concerned. Typical responses to a supplement in a given series are presented so that the weight gain can be compared to that for the control group of that series. Each weight listed in the table is the average of at least six chicks in a group. Each supplement was tested in at least three different series of experiments. The pertinent data are summarized in table 2.

TABLE 2

Typical growth responses by chicks fed various supplements together with the heated diet.[1]

SUPPLEMENTS TO 100 GM. OF RATION 241 H.	AVERAGE WT. IN 4 WEEKS
Factor U	
1.5 mg. P.A.[2]	102
Factor U + 1.5 mg. P.A.	128
Cartilage	
20% cartilage	66[3]
1.5 mg. P.A.	101
1.5 mg. P.A. + 20% cartilage	100[4]
1.5 mg. P.A. + factor U	124[4]
1.5 mg. P.A. + factor U + 10% cartilage	163[4]
1.5 mg. P.A. + 3% S.L.E.[5] +10% cartilage	164[4]
1.5 mg. P.A. + 3% S.L.E. + 0.5% cystine + 0.5% arginine + 3% glycine	152[4]
Biotin	
5 or 10% molasses	79[3]
1.5 mg. P.A.	124
1.5 mg. P.A. + 10% molasses	133
1.5 mg. P.A. + factor U + 10% molasses	169
5% liver residue	87
5% L.R. + 1.5 mg. P.A.	177
5% heated L.R. + 1.5 mg. P.A.	125
1.5 mg. P.A. + 3% S.L.E. + 10% cartilage	164 (210)[4]
1.5 mg. P.A. + 3% S.L.E. + 10% cartilage + 5% L.R.	213 (280)
1.5 mg. P.A. + 3% S.L.E. + 10% cartilage + 15 μg. biotin conc.	205 (273)
Para-aminobenzoic acid and vitamin K	
241H + 1.5 mg. P.A. + 40 mg. PABA	133[4]
1241H[6] basal	37[3]
1241H + 5 mg. vitamin K	39[3]
1241H + 1.5 mg. P.A. + 5 mg. vitamin K	47
1241H + 15 mg. P.A. + 40 mg. PABA + 5 mg. vitamin K	52
Inositol	
1.5 mg. P.A.	132
1.5 mg. P.A. + 100 mg. inositol	137
1.5 mg. P.A. + 40 mg. PABA +100 mg. inositol	151
All factors	
1.5 mg. P.A. + 3% S.L.E. +10% molasses + 10% cartilage	170
1.5 mg. P.A. + 10% molasses +100 mg. inositol + 10% cartilage	168[4]
1.5 mg. P.A. + 3% S.L.E. + 100 mg. inositol + 10% cartilage	165[4]
1.5 mg. P.A. + 10% molasses +10% cartilage + 100 mg. inositol + 3% S.L.E.	193
1.5 mg. P.A. + 10% molasses + 3% S.L.E. + 100 mg. inositol	154[4]
1.5 mg. P.A. + 3% S.L.E. + 10% cartilage + 15 μg. biotin	210

[1] Figures within parentheses give average weights of the chicks when 5 weeks of age.

[2] Calcium pantothenate.

[3] Pantothenic acid deficiency symptoms.

[4] Biotin deficiency symptoms.

[5] Solubilized liver extract, a source of factor U.

[6] One hundred-hour dry heating of ration (120°C.).

Factor U

Stokstad and Manning ('38) first attributed growth responses in chicks to a methyl alcohol-water soluble fraction of yeast. This soluble fraction which was then treated with fuller's earth and the eluate obtained by treatment with mild alkali, was called "Factor U." When this eluate fraction was added to our heated diet at a level equivalent to 10% yeast a definite growth response over that obtained with adequate quantities of calcium pantothenate alone resulted. In our more recent trials a "solubilized liver extract" was used as the source of the factors in the factor U fraction of yeast. At 4 weeks of age the chicks receiving calcium pantothenate plus this preparation were on the average 25 to 30 gm. heavier than those on the basal diet. The effect of the added factor U was observed in several instances where it was added (1) with calcium pantothenate alone, (2) with calcium pantothenate and molasses, or (3) with calcium pantothenate and cartilage. The factor U preparation was found to have little effect on the growth of chicks fed the basal diet with no added calcium pantothenate. It is evident that calcium pantothenate must be included before any study can be made of additional factors required by chicks fed this diet.

Cartilage growth factor

The necessity of supplying the "cartilage growth factor" to purified rations for chicks in order to obtain increased growth has been pointed out by Hegsted et al. (Hegsted, Hier, Elvehjem and Hart, '41). It has been shown (Almquist, Stokstad, Mecchi and Manning, '40; Almquist and Mecchi, '40) that chicks require glycine and certain carbohydrates for normal growth, and Hegsted, Briggs, Elvehjem and Hart ('41) have shown that arginine produces marked responses in chicks fed simplified diets. Arginine and glycine are able to replace cartilage to a large extent when fed together, and are able to give better feathering as well as improved growth. It was previously shown by Arnold et al. ('36) that the arginine

requirement of the chick is greater than can be supplied by 18 parts of casein. Although the addition of 10% casein did not improve the growth of our chicks maintained on the heated diet, it did appear advisable to feed cartilage both as a source of certain amino acids and for other factors which exist in this preparation. When either 10 or 20% cartilage was added to the basal diet there was no growth response whatever, and the addition of either 10 or 20% cartilage to the ration containing adequate calcium pantothenate also failed to produce a response when compared with that obtained on the ration containing calcium pantothenate alone. The marked response obtained when 10% cartilage is added to the ration supplemented with calcium pantothenate and a factor U concentrate contrasts sharply with the growth obtained with pantothenic acid and factor U alone. It appears then that the limiting deficiency in the heated diet in addition to pantothenic acid is one or more of the factors contained in the factor U preparation. Only after this deficiency is satisfied can the growth effect due to cartilage be obtained. On the basis of chemical properties and the method of preparation of the factor U fraction, it is unlikely that the cartilage factors are contained in the yeast fraction. The addition of 1% arginine, 1% cystine and 3% glycine gave somewhat less growth than 10% cartilage when the factor U was supplied by the solubilized liver residue.

Biotin

Throughout several years of work on this ration we had observed in chicks receiving the basal heated diet marked incrustations at the corners of the mouth, and scaliness and fissuring on the bottom of the feet and in between the toes. When adequate calcium pantothenate was included in the diet, the incrustations at the corners of the beak were absent while the dry scaly callousness on the bottom of the feet persisted. This scaly dermatitis was similar to that described by Hegsted et al. ('40).

We had observed in several series of experiments that both liver residue and molasses were able to prevent the leg

dermatitis. Subsequent work conclusively demonstrated to us that the active factor in these materials was biotin. When liver residue, which is the insoluble material remaining after the hot water extraction of fresh liver, was fed at a 5% level plus calcium pantothenate, there was obtained a definite response in growth together with the complete prevention of all dermatitis. The liver residue was undoubtedly able to supply sufficient factor U and biotin to account for the growth obtained. When 10% molasses was fed, enough biotin was present for the prevention of the leg dermatitis but growth was limited by the absence of factor U.

When the liver residue was heated for 30 hours at 120°C. in a manner similar to the heat treatment of the basal ration, a decrease in growth resulted, as can be seen from table 2. In most cases heating the liver residue reduced the growth response by nearly one-third, and this was an indication that it contained a heat labile factor which was destroyed by dry heat treatment. It would appear that this heat labile factor is related to one of the factors in the factor U preparation since good growth was again obtained if heated liver residue was fed together with solubilized liver extract.

Several groups of chicks were fed biotin [2] at levels of 15 μg. per 100 gm. of the ration with different combinations of the supplements. No scaly dermatitis was observed in the groups receiving the biotin and again growth was very much improved. The report of Ansbacher and Landy ('41) cited the fact that as little as 1.25 μg. of biotin per day by either oral, subcutaneous or intramuscular administration was able to cure the dermatosis in their chicks maintained on a diet similar to ours but heated for a much longer period. Our chicks received at least 1.5 μg. per day if it is granted that the birds ate an average of 10 gm. of feed a day. An important difference between the work of Ansbacher and Landy and this report is that our diet is more complete and thus accounts for the better growth of our chicks. It is quite possible therefore that the increased weight of our birds demanded a greater

[2] Obtained from SMA Corp., Chagrin Falls, Ohio.

quantity of biotin than was supplied by 10% molasses. Patrick et al. ('41) found that biotin prevented the dermatitis in turkey poults under their conditions rather than riboflavin as reported by Jukes ('38). The importance of considering the detailed procedure and conditions of each investigator in explaining the results obtained cannot be overemphasized. Hegsted et al. ('42) have fixed the biotin requirement of the chick at 7–10 μg. per 100 gm. of a purified diet. We have made no attempt to establish the biotin requirement of chicks fed the heated diet.

Para-aminobenzoic acid, vitamin K and inositol

It has been reported by Ansbacher ('41 a, '41 b) that both vitamin K and para-aminobenzoic acid (PABA) produce growth responses on a 168-hour heated diet. All our attempts to confirm this work by using a 100-hour heated (120°C.) basal diet in which vitamin K was omitted have met with failure. Levels of 40 mg. PABA per 100 gm. of diet did not give any growth response over that obtained with adequate levels of calcium pantothenate. Addition of vitamin K in the form of 2-methyl,-1,4-naphthoquinone was also ineffective at the levels fed. When both vitamin K and PABA were added to the diet no increase in the weight of the chicks was observed beyond that due to pantothenic acid alone.

The addition of PABA to the 30-hour heated diet was also ineffective in increasing the growth obtained beyond that due to additions of calcium pantothenate alone. The growth response with pantothenic acid on the excessively heated diet of Ansbacher was also less than on the ordinary heated diet and this may be ascribed to destruction of several additional factors by the prolonged heat treatment. We must conclude that under our conditions no response was obtained in chicks fed either vitamin K or PABA. It would seem appropriate to retest the effect of PABA and vitamin K on the heated diet when the latter is supplemented with those limiting factors described in this report.

Hegsted, Briggs, Mills, Elvehjem and Hart ('41) have presented some preliminary data which indicate that the growing

chick requires inositol for normal nutrition. Inositol was fed as a supplement to the heated diet at a 0.1% level both alone and together with other factors. Experiments with twenty or more groups of chicks have presented data which would indicate that under our conditions, using the heated ration, there is no growth effect with inositol, probably due to the fact that the phytin in the grains of our diet is not destroyed by the heat treatment.

DISCUSSION

The data obtained from our experiments clearly indicate that the amount of calcium pantothenate required for complete prevention of pantothenic acid deficiency dermatitis in chicks is approximately one-half that necessary for maximum growth on the heated diet. The data also show that a factor or group of factors contained in the factor U concentrate from yeast or liver is required by growing chicks fed the heated diet. The addition of cartilage was without effect when added alone, but in conjunction with factor U supplements there was a marked increase in growth. The added growth due to factor U and to cartilage was only obtained when the primary deficiency, namely, lack of pantothenic acid, was completely prevented. Repeated trials have conclusively proved that addition of individual growth factor concentrates to the basal ration gave growth responses which were directly correlated to the pantothenic acid content of the supplements.

The data indicate that added biotin is required for growth by chicks maintained on the heated diet. The dermatitis which develops primarily on the legs is usually seen in those birds which have received a ration supplemented with various concentrates and which have been maintained on the diet beyond 4 weeks. Good growth is evidently necessary in order to produce the symptoms in the chick. The growth responses due to molasses and liver residue were at first believed to be due to several substances in these materials, but later our experiments definitely indicated that biotin alone could account for the increased growth obtained. That biotin was concerned

in the growth response and in the prevention of scaly dermatitis by liver residue was further demonstrated in the complete lack of leg dermatitis in those chicks receiving this supplement.

The dry heating of the grain and casein ration evidently destroys the biotin as well as the pantothenic acid and factor U present in these natural materials; growth is better and no dermatitis is observed in chicks fed the unheated diet. It was of interest therefore to note that heating the liver residue in a manner similar to that of the basal diet caused a greater incidence of dermatitis together with a decrease in the growth response. The decrease due to the heating seemed to be less marked when the diet was improved by the addition of several growth factor concentrates. The greatest differences were observed when only calcium pantothenate was included with the heated and unheated liver residue.

It is our impression from the data here presented that liver residue contains both biotin and factor U. Certain preliminary evidence (Hutchings et al., '41) has indicated that a chick eluate factor (factor U) and a bacterial growth factor (since named "folic acid" by Mitchell et al. ('41)) are identical. It is presumed by us that in addition to biotin the other heat labile factor in liver residue may be identical with "folic acid" or one of the factors in the factor U fraction.

Under the conditions of our experiments we have not been able to substantiate the claims of Ansbacher ('41 a) that para-aminobenzoic acid is required by chicks maintained on the heated diet. The data would also indicate that inositol was without any activity when added to the heated diet.

Nearly optimum growth was obtained when the heated ration was supplemented with biotin, cartilage, factor U and pantothenic acid. When any of the four supplements was omitted the growth did not compare favorably with that obtained when all the fractions were supplied. The addition of 5% of yeast or 3% of whole liver did not produce better growth than the combination of the fractions.

SUMMARY

In addition to the six crystalline factors of the B group of vitamins, growing chicks maintained on the heated diet require the eluate factor in yeast (factor U), cartilage, and biotin. Factor U and biotin may be supplied by liver residue. Pantothenic acid, biotin and one or more factors in the factor U fraction are destroyed by the dry heat treatment.

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